Supreme Court, U.S.
FILED

(1)

04 695 NOV 22 2004

OFFICE OF THE CLERK

No. 04-___

IN THE

Supreme Court of the United States

HOUSEY PHARMACEUTICALS, INC.,
Petitioner,

v.

MERCK & CO., INC., ASTRAZENECA UK LTD., ASTRAZENECA LP, ASTRAZENECA PHARMACEUTICALS LP, AVENTIS PHARMACEUTICALS, INC., BRISTOL-MYERS SQUIBB COMPANY, ROCHE HOLDINGS, INC., HOFFMANN-LA ROCHE INC., ROCHE LABORATORIES, INC., SYNTEX (U.S.A.) INC., WYETH, AND WYETH PHARMACEUTICALS INC.,
Respondents.

Petition for a Writ of Certiorari to the United States Court of Appeals for the Federal Circuit

---

PETITION FOR A WRIT OF CERTIORARI

R. TERRANCE RADER
JAMES F. KAMP
RADER, FISHMAN & GRAUER PLLC
39533 Woodward Ave.
Suite 140
Bloomfield Hills, MI 48304
(248) 594-0600

RICHARD G. TARANTO
(Counsel of Record)
FARR & TARANTO
1220 19th Street, NW
Washington, DC 20036
(202) 775-0184

WILSON-EPES PRINTING CO., INC. – (202) 789-0096 – WASHINGTON, D. C. 20001

QUESTION PRESENTED

Whether the construction of patent claims should be governed by a substance-driven focus on sensibly interpreting claim language so as to capture the advances over prior art, understood from the context of the whole patent document and prosecution history, and avoiding constructions that invalidate the patent where reasonably possible.

TABLE OF CONTENTS

	Page
QUESTION PRESENTED	i
TABLE OF AUTHORITIES	iv
OPINIONS BELOW	1
JURISDICTION	1
STATEMENT	1
A. Background	1
B. The Patents	3
C. District Court Proceedings	7
D. The Court of Appeals Decision	8
REASONS FOR GRANTING THE PETITION	12
I. THE PROPER APPROACH TO PATENT CLAIM CONSTRUCTION IS INDISPUTABLY IN NEED OF CLARIFICATION	14
II. THIS COURT'S PRECEDENTS SUPPLY PROPER CLAIM CONSTRUCTION PRINCIPLES	17
III. THIS CASE IS A GOOD VEHICLE FOR ESTABLISHING THE PROPER SUBSTANCE-FOCUSED, SENSE-DRIVEN, CONTEXT-DEPENDENT APPROACH TO CLAIM CONSTRUCTION	22
CONCLUSION	30

TABLE OF AUTHORITIES

CASES	Page
ACS Hospital Sys., Inc. v. Montefiore Hosp., 732 F.2d 1572 (Fed. Cir. 1984)	14
AK Steel Corp. v. Sollac & Ugine, 344 F.3d 1234 (Fed. Cir. 2003)	15
Bates v. Coe, 98 U.S. 31 (1878)	17
Bell Atlantic Network Serv., Inc. v. Covad Communications Group, Inc., 262 F.3d 1258 (Fed. Cir. 2001)	20
C.R. Bard, Inc. v. United States Surgical Corp., 2004 WL 2414612 (Fed. Cir. Oct. 29, 2004)	15, 20
Carman Indus., Inc. v. Wahl, 724 F.2d 932 (Fed. Cir. 1983)	14
Eastman Kodak Co. v. Goodyear Tire & Rubber Co, 114 F.3d 1547 (Fed. Cir. 1997) *limited in other respect, Cybor Corp. v. FAS Technologies, Inc.*, 138 F.3d 1448 (Fed. Cir. 1998)	14
Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki Co., 535 U.S. 722 (2002)	13, 19
Gaus v. Conair Corp., 125 S. Ct. 346 (2004)	30
Generation II Orthotics Inc. v. Med. Tech. Inc., 263 F.3d 1356 (Fed. Cir. 2001)	15
Graham v. John Deere Co., 383 U.S. 1 (1966)	20
Harris Corp. v. IXYS Corp., 114 F.3d 1149 (Fed. Cir. 1997)	14, 23
Honeywell v. Victor Co. of Japan, Ltd., 298 F.3d 1317 (Fed. Cir. 2002)	21
Irdeto Access, Inc. v. Echostar Satellite Corp., 383 F.3d 1295 (Fed. Cir. 2004)	20
Klein v. Russell, 86 U.S. (19 Wall.) 433 (1874)	18
Lawrence v. Chater, 516 U.S. 163 (1996)	30
Liebel-Flarsheim Co. v. Medrad, Inc., 358 F.3d 898 (Fed. Cir.), *cert. denied*, 125 S. Ct. 316 (2004)	15, 20

TABLE OF AUTHORITIES—Continued

Page

Markman v. Westview Instruments, Inc., 517 U.S. 370 (1996) ..... *passim*
Microsoft Corp. v. Multi-Tech Sys., Inc., 357 F.3d 1340 (Fed. Cir.), *cert. denied*, 125 S. Ct. 61 (2004) 20, 30
Minnesota Mining & Mfg. Co. v. Johnson & Johnson Orthopaedics, Inc., 976 F.2d 1559 (Fed. Cir. 1992) 14
On-Line Technologies, Inc. v. Bodenseewerk Perkin-Elmer GmbH, 386 F.3d 1133 (Fed. Cir. 2004) 20, 22
Phillips v. AWH Corp., 376 F.3d 1382 (Fed. Cir. 2004) 12, 15, 19
Rambus Inc. v. Infineon Technologies AG, 318 F.3d 1081 (Fed. Cir. 2003) 20, 21
SciMed Life Sys., Inc. v. Advanced Cardiovascular Sys., Inc., 242 F.3d 1337 (Fed. Cir. 2001) 20
Smith v. Snow, 294 U.S. 1 (1935) 18
Texas Instruments Inc. v. ITC, 988 F.2d 1165 (Fed. Cir. 1993) 14
Turrill v. Michigan S. & N.I.R.R., 68 U.S. (1 Wall.) (1864) 18
United States v. Adams, 383 U.S. 39 (1966) 12, 18
Unique Concepts, Inc. v. Brown, 939 F.2d 1559 (Fed. Cir. 1991) 14
Warner-Jenkinson Co., Inc. v. Hilton Davis Chem. Co., 520 U.S. 17 (1997) 13, 19
Whittaker Corp. v. UNR Indus., Inc., 911 F.2d 709 (Fed. Cir. 1990) 15

TABLE OF AUTHORITIES—Continued

OTHER MATERIALS	Page
28 U.S.C. § 1254(1)	1
35 U.S.C. § 112, ¶ 1	16, 20
§ 282	18
37 C.F.R. § 1.175(d)(1)	21
B. Alberts *et al., Molecular Biology of the Cell* (4th ed. 2002)	3, 28
"Beating Cancer: The New Frontier of Molecular Medicine," *The Economist*, Oct. 16, 2004	2
Buchdunger *et al., Inhibition of the Abl Protein-Tyrosine Kinase* in Vitro *and* in Vivo *by a 2-Phenylaminopyrimidine Derivative,* 56 Cancer Research 100 (1996)	3
IBM et al. Amicus Brief, *Phillips v. AWH Corp.* (Fed. Cir. 2004), www.patentlyobviousblog.com/files/IBM.pdf	21
United States Amicus Brief, *Phillips v. AWH Corp.* (Fed. Cir. 2004), www.patentlyobvious blog.com/files/govt_brief.pdf	21

PETITION FOR A WRIT OF CERTIORARI

Housey Pharmaceuticals, Inc. (Housey), petitions for a writ of certiorari to review the judgment of the United States Court of Appeals for the Federal Circuit in this case.[1]

OPINIONS BELOW

The Federal Circuit's opinion (Pet. App. 1a-22a) is reported at 366 F.3d 1348. The district court's opinion on claim construction (Pet. App. 23a, 30a-32a) is not reported.

JURISDICTION

The judgment of the court of appeals was entered on May 7, 2004. Pet. App. 1a. A timely petition for panel and en banc rehearing was denied on August 25, 2004. Pet. App. 33a. This Court has jurisdiction under 28 U.S.C. § 1254(1).

STATEMENT

A. Background

This case involves patents for a method of sifting through substances to see which ones have a particular desirable property—the ability to inhibit or activate a specific pre-identified protein in a cell. The most prominent focus of the patented method is on proteins that have already been recognized to be responsible for the uncontrolled growth that characterizes cancer cells. If substances can be identified that target such a particular protein, they offer the possibility, if turned into therapeutic drugs, of turning off the cancerous growth without the adverse collateral harms to other cells (such as other fast-growing healthy cells) that are caused by cruder therapeutic substances, which attack cancerous growth by means other than targeting the responsible protein. The patented method at issue seeks to discover such specific protein-targeting substances by using a cell line that over-produces the target protein (relative to a control line of cells),

---

[1] Pursuant to Rule 29.6, Housey states that it has no parent corporation, and no publicly held company owns 10% or more of its stock.

noticing how a pre-identified cellular property correlates with the increased level of protein (the patents call this a "graded cellular response"), then exposing the protein-overproducing cells and the control line to test substances, and observing whether the substances differentially affect the pre-identified protein-correlated cellular property in the two cell lines.

The new significance of substances that inhibit or activate a particular protein of interest is well explained, for the cancer context, in the recent cover story in *The Economist*, Oct. 16, 2004, at 75 (cover: "Beating cancer: The new frontier of molecular medicine"). "Cancer is characterised by uncontrolled cell growth." *Id.* "Most current therapies—drugs that were developed during the first golden era, in the 1970s—simply attack dividing cells. That stops tumours, but it also kills all rapidly proliferating cells, regardless of their origin, function or state of health. The action of these drugs on normal tissues causes the side effects, including nausea, fatigue and weakened immune systems, associated with cancer treatment. By contrast, the new generation of drugs . . . attack only deranged cells. These drugs are designed to fix the specific molecular problems in a tumour that drive its growth. [¶] The best known of these new drugs is Gleevec[,] . . . designed to halt a blood cancer called chronic myeloid leukaemia (CML)," which is caused by a chromosonal abnormality that produces a "novel protein . . . known as BCR-ABL," "one of a class of enzymes known as tyrosine kinases," which induces cancerous growth. *Id.* "Gleevec blocks the activity of BCR-ABL *by sitting in the enzyme's active site.* This switches off the signal to grow, and the cancer stops proliferating." *Id.* at 76 (emphasis added to indicate that Gleevec binds to the protein). In short, finding substances that selectively bind to identified proteins, and thereby block their activity, is increasingly important, espe-

cially in cancer research. *See* B. Alberts *et al.*, *Molecular Biology of the Cell* 1358-60 (4th ed. 2002).[2]

B. The Patents

Four patents, with Dr. Gerard Housey, MD, PhD, as inventor and Housey Pharmaceuticals now as assignee, are at issue. Pet. App. 2a. All are entitled, "Method of Screening for Protein Inhibitors and Activators." They describe "a general screening method for the *discovery* and *identification* of both inhibitors and activators of enzymes, receptors, and other proteins"—particularly, "a method of screening for substances which *specifically* inhibit or activate a particular protein affecting the cultural or morphological characteristics of the cell expressing the protein . . . ." Patent No. 4,980,281, Col. 1, ll. 7-14 (emphases added).[3] The patents take as already understood the defining characteristic of the substances being sought (inhibitors and activators of a pre-identified protein): the already-understood defining characteristic is, of course, the reason for wanting to find them. The patents then lay out a method of finding such substances—of "discover[ing]" them, of "identif[ying]" them, of "screening for" them.

The specification repeatedly explains that the invention picks out, from among the entire class of substances that affect a particular cell property (*e.g.*, its cancerous growth), the substances that do so by acting *on the protein*, distinguishing

---

[2] Gleevec itself is made by Novartis, a licensee of Housey's, whose method of cellular screening with protein-overproducing cells was used to identify the active ingredient in Gleevec as an inhibitor of the protein kinase associated with chronic myelogenous leukemia. *See* Buchdunger *et al.*, *Inhibition of the Abl Protein-Tyrosine Kinase* in Vitro *and* in Vivo *by a 2-Phenylaminopyrimidine Derivative*, 56 Cancer Research 100 (1996); Alberts, *supra*, at 1359-60 (figure shows Gleevec binding). Most of the world's major pharmaceutical companies have licensed the patents at issue.

[3] All citations to the patents, unless otherwise indicated, are to Patent No. 4,980,281, given by column and line numbers.

them from substances that affect the cell property by acting on *other components of the cell*. It achieves this critical experimental separation by the observed differential effect on a carefully selected "phenotypic characteristic" that has been confirmed, before the substance-testing step of the method, as correlating with increasing expression of a "protein of interest" (POI). Col. 2, ll. 40-42, 60-62; Col. 6, ll. 14-17.[4] Thus, the patented method uses the "greater effect on the test lines than on the control lines" (Col. 3, ll. 9-10), the "greater effect on the phenotype of the higher producing cell line" (Col. 4, ll. 49-50), to "distinguish[]" (Col. 3, l. 7), to "identif[y]" (Col. 4, l. 48), the desired subgroup among all cell-property-affecting substances. It is not too much to say that the making of this separation is the essence of this invention.

Immediately after the general "Field of the Invention" para graph (Col. 1, ll. 7-15), the specification describes prior-art assays (screening methods) for the crucial purpose of identifying the inventive contribution here—the advance over the prior art. Col. 1, l. 16-Col. 2, l. 22. It notes several prior-art "assay systems" used "for the discovery of new modulators of cell growth, and in particular, in the search for new anti-cancer drugs which are specifically toxic to cancer cells but not to normal cells." Col. 1, ll. 17-21. Thus, "[i]n vitro cytotoxicity assays" (lab-dish testing for toxicity to cells) measure "the inhibition of certain cellular metabolic pathways in response to treatment with cytotoxic agents," but because what is tested is inhibition of the overall multi-step "pathways" through which some cellular process (metabolism) takes place, without being limited to the targeted blocking of a selected protein within such pathways, they "are

---

[4] In biology, a "phenotype" is contrasted with a "genotype." A "genotype" is the *genetic* constitution of a cell or organism. "Phenotype" is the broad, catchall term embracing the set of discernable features of the cell or organism (generated by its genetic code as it interacts with its environment)—including its growth rate, shape ("morphology"), and other characteristics.

non-specific since any agent which alters the growth properties of cells will score positively in these assay systems." Col. 1, ll. 28, 31-33, 38-40. Again, certain prior-art assays conducted in (gelatin-like) soft agar are "also non-specific" because "any agent which inhibits cellular growth *in any way* will score[] positively in this assay system as it is currently used, whether or not it inhibits the protein of interest." Col. 1, ll. 51-59 (emphasis added). The specification also describes another prior-art method (Uehara) as "currently the most effective one for developing specific agents which [either] interact with a given protein *or* alter a specific cellular property" (Col. 2, ll. 12-14 (emphasis added; "either" inserted for emphasis)). In all these descriptions, the specification identifies the limitation of the pre-existing methods: they merely find substances that can modulate a cellular property of interest (*e.g.*, cancerous growth); they do not sift among such substances to find those which achieve that effect by specifically interacting with an identified protein, rather than through other mechanisms.

Having identified the limiting character of prior-art methods, the specification several times pinpoints that the new method makes the very separation prior-art methods do not. Thus, it immediately distinguishes the new method as exceeding the "specificity" of the just-described Uehara method (Col. 2, ll. 26-27) and declares that the new method involves

> the generation of a cell line purposefully engineered to detect both stimulatory and inhibitory agents which are *absolutely specific for any given protein* which affects the cultural or morphological characteristics of the cell.

Col. 2, ll. 29-33 (emphasis added). Distinguishing among cell-property-altering substances, the specification says that the claimed assay tells investigators which kind they have found: "Substances which *specifically inhibit or []activate the POI* may be distinguished from substances which affect cell morphology or growth *by other mechanisms* in that they

will have a greater effect on the test lines than on the control lines." Col. 3, ll. 5-10 (emphasis added). After describing the basic idea of creating a POI-overproducing cell line, and calling for any kind of "phenotypic characteristic" that correlates with the level of that protein (Col. 4, l. 51-Col. 6, l. 13), the specification again stresses the crucial distinction among ways of altering cell properties (Col. 6, ll. 14-20 (emphasis added)):

> What we are looking for is a increase in the phenotypic change exhibited by the cell which becomes greater with increasing expression of the POI. We call this a "graded cellular response," and it is by this specialized response that *we distinguish inhibitors or activators of the POI from agents that act upon other cell metabolites to effect a phenotypic change.*

Much of the rest of the specification is taken up with details showing how to prepare protein-overproducing cells—mostly focused on the cancer-causing protein kinase C (PKC), an enzyme—and to identify phenotypic characteristics correlated with the protein level (*e.g.*, formation of clumps called foci as cells grow, the ability to grow suspended in a gelatinous medium called soft agar). Near the end, the specification reports certain experiments and says (Col. 18, ll. 31-36 (emphasis added)):

> this work establishes, for the first time, the fact that stable overproduction of a protein in mammalian cells can result in a novel cellular phenotype(s) (in this case anchorage independence) which can be *directly modulated by chemical agents which interact with the protein.*

Claim 1 of the earliest patent, the '281 patent, claims a "method of determining whether a substance is an inhibitor or activator of a protein." The method includes ("comprises," in patent language) the steps of taking two cell lines carefully differentiated by the level of a specified protein, exposing them to a substance one is trying to test, and observing whether the test substance has a differential effect

on a pre-selected property ("phenotypic characteristic") of the cell lines that has been correlated with the protein level. *See* Pet. App. 3a-4a.

> 1. A method of determining whether a substance is an inhibitor or activator of a protein whose production by a cell evokes a responsive change in a phenotypic characteristic other than the level of said protein in said cell per se, which comprises:
>
> (a) providing a first cell line which produces said protein and exhibits said phenotypic response to the protein;
>
> (b) providing a second cell line which produces the protein at a lower level than the first cell line, or does not produce[] the protein at all, and which exhibits said phenotypic response to the protein to a lesser degree or not at all;
>
> (c) incubating the substance with the first and second cell lines; and
>
> (d) comparing the phenotypic response of the first cell line to the substance with the phenotypic response of the second cell line to the substance.

One dependent claim, '281 Patent, Claim 8, claims that method applied particularly to an enzyme. Other claims in the patents claim variations on that basic method.

C. District Court Proceedings

Housey brought a patent infringement action against respondents. For purposes of construing the claims at issue, the district court proceedings were consolidated with an earlier declaratory-judgment action brought by Bayer AG against Housey. *See* Pet. App. 2a. The district court rendered constructions of several patent claim terms in the Bayer case, constructions that also applied in this case. Pet. App. 23a, 30a-32a. The court concluded, in particular, that an "inhibitor or activator of a protein" is any "substance that has

a greater effect on the phenotype of cells that express the protein of interest at a higher level than on the phenotype of cells that express the protein of interest at a lower level or not at all." Pet. App. 31a. In other words, the court *defined* the object of search in this screening method, an "inhibitor or activator of a protein," as any substance that has the differential effect on the two cell lines that the method looks for. That construction makes the method, by definition, a 100% perfect identifier of whether a substance is an inhibitor or activator of a protein. At the same time, though, the district court construed "method of determining" *not* to require the answer of the screening method to be "100% certain," concluding instead that (as in science generally) "determining" here means "ascertaining with a high degree of probability that an unknown or suspected substance is an activator or inhibitor of a target protein." Pet. App. 32a.

A few weeks after the claim-construction ruling, respondents and Housey stipulated that the patents were invalid and not infringed under the district court's construction of "inhibitor or activator of a protein." Pet. App. 24a-29a. On that basis the court entered judgment. Pet. App. 23a.

D. The Court of Appeals Decision

A divided panel of the court of appeals affirmed the construction of "inhibitor or activator of a protein" and, therefore, the judgment. Pet. App. 1a-14a. The majority adopted the district court's construction of "inhibitor or activator of a protein" as meaning any substance that produces a greater effect on a phenotypic characteristic of the protein-overproducing cell lines than on control cells, regardless of how that effect is achieved. Pet. App. 2a, 7a. Because the stipulated judgment depended expressly on the particular construction the district court adopted, any different construction by the court of appeals would have required a remand.

The majority began by stating that "a discernable plain and ordinary meaning of the claim language . . . usually defines the scope of the claims unless the patentee has explicitly disclaimed or clearly disavowed this meaning in the specification or prosecution history." Pet. App. 6a-7a. The majority then analyzed this case. In two paragraphs it drew a conclusion about the proper meaning from the claim language itself and two statements in the specification—without considering what sense this construction made, the overall context of the patent documents to determine how to capture the advance over prior art, or the stipulated invalidity under that construction. Pet. App. 7a-8a.

The majority drew its conclusion from the "language of claim 1 of the '281 patent," which it declared was "inimical" to any different construction. Pet. App. 7a. It concluded, simply, that a substance that has a differential effect on the two cell lines "is, as *defined by* the claimed method itself, an inhibitor or activator of the protein of interest." *Id.* (emphasis added). Against the backdrop of its conclusion that the claim language was "inimical to any narrower construction," the majority said that "inhibitor or activator of a protein" was "expressly define[d]" by statements in the specification that such substances "are *identified* by their" differential effect on the two cell lines (Col. 4, ll. 48-50) and can be "*distinguished* from substances which affect cell morphology or growth by other mechanisms in that they will have greater effect on the test lines than on the control lines" (Col. 3, ll. 5-10). Pet. App. 7a-8a (emphases added). The majority rejected Housey's central contention that this construction confuses a high-probability way of finding (distinguishing, identifying) "inhibitors or activators of a protein" with the *definition* of what one is looking for. Pet. App. 8a. The majority reasoned that, because the claim "addresses a method of determining whether a compound is an inhibitor or activator that is conducted by observing whether a particular event occurs when the method is performed," it is "logical to

construe an 'inhibitor or activator of a protein' as a substance that precipitates that event." *Id.*

The foregoing reasoning is the sum total of the support the majority offered for its construction. The remainder of its opinion addresses only the merits of Housey's construction. Housey proposed that "inhibitor or activator of a protein" refers to substances that achieve their modulating effect on the cellular activity of the protein by binding to the protein (or the multi-part molecular complex of which it is a part, as the prosecution history explains), not by other means. The majority offered several reasons for rejecting that construction.

As to claim language, the majority recognized that standard technical dictionaries include definitions of "activator" and "inhibitor" for "enzyme" that, as Housey urged, "include a direct-binding limitation." Pet. App. 9a. The majority noted that the dictionary definitions are written in terms of inhibitors/activators of "enzymes," but did not explain why the same definitions are not available for proteins generally. And the majority asked only whether those definitions established "the plain meaning," not whether they established *available* meanings (*id.*)—a critical reformulation, because if binding were even a permissible meaning, the specification and prosecution history would supply the context for discerning the right meaning, with no burden to overcome a presumptively clear contrary meaning. *Id.*

As to the specification, the majority never discussed key aspects—those, quoted above, which focus the patented method's advance over prior art on its distinguishing among cell-property-affecting substances based on the mechanisms of achieving that effect. In particular, the majority made no mention of the specification's emphasis on the absence of that distinguishing ability as a fundamental limitation of prior-art methods. The majority did recognize the specification's repeated emphasis that the method identifies activators or inhibitors "specific" or "absolutely specific" to a target protein (Pet. App. 11a-12a), but it deemed those passages insufficient

to "lead to a strong inference of direct binding." *Id.* at 12a. It never explained how those passages could be squared with the construction *it* adopted, however, and it did not discuss the evidence, partly set forth in the dissent (*id.* at 22a), that a number of experts, including one adversarial to Housey, understood "specific" to refer to binding.

Beyond explaining away material urged by Housey as supporting its construction, the majority opinion relies on just a few passages as affirmatively pointing the other way. The majority cited three passages in the specification (Pet. App. 10a, 11a), two of which (Col. 6, ll. 37-40, 40-42) on their face are not even addressed to *how* cellular properties are altered (by binding to the protein or by some other action in a biological pathway), but only to *how much* alteration is required. (The third is discussed at page 27, *infra*.) The majority also relied on a passage from the prosecution history about the standard situation where a protein resides within a molecular complex, reading the passage as allowing for inhibitors or activators to affect any aspect of a particular biological pathway. Pet. App. 11a. That is a misreading, as discussed at pages 28-29, *infra*.

Finally, the majority dismissed the venerable principle that patent claims should be "'so construed, if possible, as to sustain their validity.'" Pet. App. 14a. The majority said that, unless it was itself determining validity, the principle was inapplicable—despite respondents' *stipulation* that their proposed construction invalidates the patents. *Id.* In any event, the majority said in a single sentence that the principle was not "dispositive" here, because it had already determined the proper construction without regard to that principle. *Id.*

Judge Newman dissented. Pet. App. 15a-22a. She disagreed with what she described as the majority's treatment of the specification as "an also-ran in claim construction," when it should be "the primary resource in understanding the claims." Pet. App. 17a; *id.* at 15a-19a. More pointedly, "the

majority's proposed 'plain meaning' differs from the specification's statement of the invention." Pet. App. 19a. In particular, on the crucial matter of identifying the advance over prior art, "[t]he patent documents state that the invention concerns 'direct modulation,' and that the prior art shows primarily 'indirect modulation' of intermediate agents." *Id.* Judge Newman quoted expert testimony, stressed the importance of construing claims to avoid invalidity where reasonably possible, and urged a remand "for resolution of conflicting scientific evidence, and re-determination of the meaning and scope of the correctly construed claims." Pet. App. 21a-22a.

REASONS FOR GRANTING THE PETITION

This case presents an opportunity for this Court to restore a focus on substance and sense to the task at the heart of the vast bulk of patent cases—claim construction, *i.e.*, the interpretation of the claims of a patent, which define the protected invention. The fundamental goal of claim construction is to "ascertain[] the invention," by reference to the entire document. *United States v. Adams,* 383 U.S. 39, 49 (1966) ("[C]laims are to be construed in the light of the specifications and both are to be read with a view to ascertaining the invention."). Yet the Federal Circuit has, in a number of cases, conducted claim construction as an almost mechanical exercise that loses sight of its proper objective: to capture the invention as accurately as possible in order to ensure a commensurate patent reward. That misguided approach destroyed Housey's patents in this case.

There is urgent need for clarification, as confirmed by the Federal Circuit's recent decision broadly to address claim-construction principles en banc. *Phillips v. AWH Corp.*, 376 F.3d 1382 (Fed. Cir. 2004). The clarification that is needed must reflect the basic recognition that, given the inevitable imperfections of language, the patent system cannot continue to fulfill its mission of spurring invention unless claim con-

struction is returned to a more context-dependent, realistic inquiry to resolve genuine linguistic uncertainty in favor of a sensible definition of what the patentee invented. Clarification along just those lines is the natural next step following this Court's recent decisions resolving matters fundamental to patent law based on appreciation of the difficulties of patent language and the need for context-dependent interpretation. *See Markman v. Westview Instruments, Inc.*, 517 U.S. 370, 389 (1996); *Warner-Jenkinson Co., Inc. v. Hilton Davis Chem. Co.*, 520 U.S. 17, 34-35 (1997); *Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki Co.*, 535 U.S. 722, 731-32 (2002). Nothing is more fundamental in patent law than the proper construction of claim language, which affects both validity and infringement issues in the great majority of patent cases.

The analysis by the panel majority in this case exemplifies claim-construction approaches that lose sight of the "does this make sense" question that should overhang, drive, and constantly inform the inquiry. The adopted construction make no sense as a definition of the invention as the patentee conceived it and the PTO understood it. Indeed, the adopted construction, by bizarrely turning a highly informative but inevitably imperfect method of screening for something into the definition of the thing being sought, renders the patent claims essentially tautological and circular, undisputedly invalidates the patents, and ignores the patent's explanation of the key point of distinction over prior-art methods. The panel majority's analysis and result in this case fall so far from the mark of capturing the actual invention that this case is a good vehicle for re-establishing a sensible realism in claim construction as the natural next step in this Court's recent course of clarifying important foundations in patent law along such lines.

I. THE PROPER APPROACH TO PATENT CLAIM CONSTRUCTION IS INDISPUTABLY IN NEED OF CLARIFICATION

Conflicts among Federal Circuit decisions establish the need for clarification of the proper approach to construing patent claims. Thus, the panel majority in this case adopted a claim construction that senselessly renders the claims tautological, effectively writing a whole clause (a claim limitation) out of the claims (pages 22-24, *infra*), whereas elsewhere the Federal Circuit has rejected claim constructions precisely where they "would make [a claim] limitation entirely circular" so as to "contribute nothing but meaningless verbiage to the definition of the claimed invention" (*Harris Corp. v. IXYS Corp.*, 114 F.3d 1149, 1152 (Fed. Cir. 1997)) or render claim language "surplusage" (*Texas Instruments Inc. v. ITC*, 988 F.2d 1165, 1171 (Fed. Cir. 1993)) or "meaningless" (*Unique Concepts, Inc. v. Brown*, 939 F.2d 1559, 1563 (Fed. Cir. 1991)). Similarly, the majority here ignored the patents' discussion of prior art limitations and how this invention overcame them (page 26, *infra*), whereas other Federal Circuit decisions rely centrally on such discussions. *E.g., Eastman Kodak Co. v. Goodyear Tire & Rubber Co.*, 114 F.3d 1547, 1554 (Fed. Cir. 1997), *limited in other respect, Cybor Corp. v. FAS Technologies, Inc.*, 138 F.3d 1448 (Fed. Cir. 1998); *Minnesota Mining & Mfg. Co. v. Johnson & Johnson Orthopaedics, Inc.*, 976 F.2d 1559, 1566 (Fed. Cir. 1992).

Federal Circuit precedents also diverge on the principle that claims should be construed to avoid invalidity where possible. A number of Federal Circuit decisions (following this Court's precedents, *see* page 18, *infra*) have relied heavily on this principle from the outset of analysis, informing the entire claim construction as it proceeds, testing the soundness of provisional conclusions along the way. *Carman Indus., Inc. v. Wahl*, 724 F.2d 932, 937 n.5 (Fed. Cir. 1983); *ACS Hospital Sys., Inc. v. Montefiore Hosp.*, 732 F.2d 1572, 1577 (Fed. Cir.

1984); *see also Whittaker Corp. v. UNR Indus., Inc.*, 911 F.2d 709, 712 (Fed. Cir. 1990). Recently, however, Federal Circuit decisions have relegated the avoid-invalidity principle to the status of an afterthought, first engaging in claim construction without regard to invalidity consequences, and only then mentioning the invalidity-avoidance principle—either making it a needless confirmation of the conclusion already reached or dismissing the principle on the ground that it cannot overcome the conclusion already reached. *E.g., Liebel-Flarsheim Co. v. Medrad, Inc.*, 358 F.3d 898, 911 (Fed. Cir.), *cert. denied*, 125 S. Ct. 316 (2004); *AK Steel Corp. v. Sollac & Ugine*, 344 F.3d 1234, 1243 (Fed. Cir. 2003); *Generation II Orthotics Inc. v. Med. Tech. Inc.*, 263 F.3d 1356, 1365 (Fed. Cir. 2001).

More broadly, the Federal Circuit has in a number of cases so veered off the course of context-dependent realism in claim construction (as the dissent explains) that the court as a whole has now formally recognized that its precedents have created widespread confusion about the proper approach to claim construction. In July 2004, the Federal Circuit granted en banc review in *Phillips v. AWH Corp.*, 376 F.3d 1382 (Fed. Cir. 2004), to consider broad questions about claim construction—particularly, the strength of any presumptive conclusion about meaning from the claim language alone, as well as the role of the principle of avoiding constructions that needlessly produce invalidity.[5] *See also C.R. Bard, Inc. v.*

---

[5] 376 F.3d at 1383: "1. Is the public notice function of patent claims better served by referencing primarily to technical and general purpose dictionaries and similar sources to interpret a claim term or by looking primarily to the patentee's use of the term in the specification? If both sources are to be consulted, in what order?

2. If dictionaries should serve as the primary source for claim interpretation, should the specification limit the full scope of claim language (as defined by the dictionaries) only when the patentee has acted as his own lexicographer or when the specification reflects a clear disclaimer of claim scope? If so, what language in the specification will satisfy those conditions? What use should be made of general as opposed to technical

dictionaries? How does the concept of ordinary meaning apply if there are multiple dictionary definitions of the same term? If the dictionary provides multiple potentially applicable definitions for a term, is it appropriate to look to the specification to determine what definition or definitions should apply?

3. If the primary source for claim construction should be the specification, what use should be made of dictionaries? Should the range of the ordinary meaning of claim language be limited to the scope of the invention disclosed in the specification, for example, when only a single embodiment is disclosed and no other indications of breadth are disclosed?

4. Instead of viewing the claim construction methodologies in the majority and dissent of the now-vacated panel decision as alternative, conflicting approaches, should the two approaches be treated as complementary methodologies such that there is a dual restriction on claim scope, and a patentee must satisfy both limiting methodologies in order to establish the claim coverage it seeks?

5. When, if ever, should claim language be narrowly construed for the sole purpose of avoiding invalidity under, *e.g.*, 35 U.S.C. §§ 102, 103 and 112?

6. What role should prosecution history and expert testimony by one of ordinary skill in the art play in determining the meaning of the disputed claim terms?

7. Consistent with the Supreme Court's decision in *Markman v. Westview Instruments, Inc.*, 517 U.S. 370 ... (1996), and our *en banc* decision in *Cybor Corp. v. FAS Technologies, Inc.*, 138 F.3d 1448 (Fed. Cir.1998), is it appropriate for this court to accord any deference to any aspect of trial court claim construction rulings? If so, on what aspects, in what circumstances, and to what extent?"

Judge Rader separately asked for comment on the following: "Is claim construction amenable to resolution by resort to strictly algorithmic rules, e.g., specification first, dictionaries first, etc.? Or is claim construction better achieved by using the order or tools relevant in each case to discern the meaning of terms according to the understanding of one of ordinary

United States Surgical Corp., 2004 WL 2414612, at *3 (Fed. Cir. Oct. 29, 2004) (describing divergent approaches within Federal Circuit decisions). The need for clarification of claim-construction principles is definitively established by the grant of en banc review in *Phillips*—and by the roughly three dozen amicus briefs, including one by the United States, that have been filed to address these fundamental questions.

II. THIS COURT'S PRECEDENTS SUPPLY PROPER CLAIM CONSTRUCTION PRINCIPLES

Under this Court's precedents, the proper approach to claim construction is one that is substance-driven, always recognizing that claims define the protected invention but looking to the entire patent document to determine how to construe the claim terms, constantly with an eye on the critical question of how sensibly to read the claims so as to capture the invention, and to avoid needlessly adopting a patent-invalidating construction. That substance- and sense-driven approach gives patents their due and provides the proper public notice of patent scope to those skilled in the art.

This Court set out the basic principles in *Bates v. Coe*, 98 U.S. 31, 38-39 (1878) (cited in *Markman*, 517 U.S. at 389). Although patentees are limited to their claims, notwithstanding greater breadth in the specification, "it is equally true that the claims of the patent, like other provisions in writing, must be reasonably construed, and in case of doubt or ambiguity it is proper in all cases to refer back to the descriptive portions of the specification to aid in solving the doubt or in ascertaining the true intent and meaning of the language employed in the claims . . . . In construing patents,

---

skill in the art at the time of the invention, thus entrusting trial courts to interpret claims as a contract or statute?" 376 F.3d at 1384.

Chief Judge Mayer opposed en banc consideration as pointless until the Federal Circuit reconsiders the rule that claim construction is subject to *de novo* review on appeal. *Id.*

it is the province of the court to determine what the subject-matter is upon the whole face of the specification and the accompanying drawings."

In *Klein v. Russell*, 86 U.S. (19 Wall.) 433, 466 (1874), the Court already had insisted that "[t]he subject is to be examined in the light of [the] specifications and of [the] sets of claims." Importantly, the Court added a vital interpretive principle: "The court should proceed in a liberal spirit, so as to sustain the patent and the construction claimed by the patentee himself, if this can be done consistently with the language which he has employed." Even that principle was not new, for the Court had earlier ruled that "[p]atents . . . are to receive a liberal construction, and under the fair application of the rule, ut res magis valeat quam pereat, are, if practicable, to be so interpreted as to uphold and not to destroy the right of the inventor." *Turrill v. Michigan S. & N.I. R.R.*, 68 U.S. (1 Wall.) 491, 510 (1864). The principle of avoiding patent-invalidating constructions, if possible, reflects the deference due the patent-issuance decision of the expert PTO—as required by Congress's command that patents "be presumed valid." 35 U.S.C. § 282.

In *Smith v. Snow*, 294 U.S. 1, 14 (1935), the Court confirmed the necessary substantive focus. It explained that "if the claim [is] fairly susceptible of two constructions, that should be adopted which will secure to the patentee his actual invention." In *United States v. Adams*, 383 U.S. at 48-49, the Court again stressed that "claims are to be construed in the light of the specifications and both are to be read with a view to ascertaining the invention."

In *Markman*, 517 U.S. at 389, the Court, in establishing claim construction as a matter for judges rather than juries, relied on *Bates* and emphasized that the task of construction was one of substance-focused understanding of the document as a whole. Claim construction requires a "necessarily sophisticated analysis of the whole document, required by the

standard construction rule that a term can be defined only in a way that comports with the instrument as a whole." *Id.* (citing *Bates* and other sources). Any proposed interpretation must be evaluated "in relation to the overall structure of the patent"; the issue is whether it "fully comports with the specification and claims and so will preserve the patent's internal coherence." *Id.* at 390.[6]

The context-dependent approach to claim construction is reinforced by other recent decisions. In *Warner-Jenkinson*, 520 U.S. at 34-35, the Court, in affirming the persistence of the doctrine of equivalents as a kind of infringement beyond the literal meaning of claim terms, recognized that the doctrine responds to the need for realism and some flexibility in protecting inventions. In *Festo*, 535 U.S. at 731-32, the Court rejected the Federal Circuit's unduly mechanical and rigid limits on the doctrine of equivalents, repudiating "literalism" in claim "interpretation" in recognition of the inevitable imprecisions of claim language. *Id.* at 731-32 (because "the nature of language makes it impossible to capture the essence of a thing in a patent application," "the clearest rule of patent interpretation, literalism, may conserve judicial resources but is not necessarily the most efficient rule").

Proper claim construction under those decisions can and should be orderly and systematic in striving for a substantively correct understanding, without being mechanical, blinkered, formulaic, "algorithmic" (Judge Rader's term in the *Phillips* grant of en banc review, 376 F.3d at 1384). For example, *if* a claim term has a clear meaning, that meaning can generate a presumption of correctness; whereas if no "precise and well-established meaning in the art" exists, the meaning, while properly limited to the range of available

---

[6] *See also* 517 U.S. at 387 (noting frequent need for non-expert judges, seeking to construe the "tenor" of a patent, to receive expert testimony to understand the meaning of technical terms).

meanings of the claim words, necessarily must be sought from context.[7] Differences among different claims' language—in the issued patent or in initial applications leading to the patent or related patents—may reveal both a broad or unmodified term, on one hand, and a narrow or modified term, on the other, suggesting a broad meaning of the former by comparison to the narrower meaning of the latter.[8] The specification can make clear an intended meaning, either by express definition or by clear, otherwise-unqualified characterization of the *invention*, especially statements distinguishing it from prior art; while specification statements that serve quite different statutory purposes—describing particular *embodiments*, to teach skilled artisans how to make and use the invention and to set out the best mode the inventor knows of practicing it (35 U.S.C. § 112, ¶ 1)—must not be transformed into limitations on the *invention*.[9] And the prosecution history, aside from showing differences among (issued or proposed) claims, can illuminate the meaning accepted by the patentee and examiner in various ways: *e.g.*, it may expressly state a narrow or broad intended meaning, or it may reveal that the examiner recognized the presence of multiple separate inventions in an initial application and required them

---

[7] *On-Line Technologies, Inc. v. Bodenseewerk Perkin-Elmer GmbH*, 386 F.3d 1133, 1137 (Fed. Cir. 2004); *Irdeto Access, Inc. v. Echostar Satellite Corp.*, 383 F.3d 1295, 1300 (Fed. Cir. 2004); *Rambus Inc. v. Infineon Technologies AG*, 318 F.3d 1081, 1088, 1094 (Fed. Cir. 2003).

[8] *Liebel-Flarsheim*, 358 F.3d at 909; *Rambus*, 318 F.3d at 1095; *see Graham v. John Deere Co.*, 383 U.S. 1, 33 (1966) ("claims that have been narrowed in order to obtain the issuance of a patent by distinguishing the prior art cannot be sustained to cover that which was previously by limitation eliminated from the patent").

[9] *C.R. Bard*, 2004 WL 2414612, at *5; *Microsoft Corp. v. Multi-Tech Sys., Inc.*, 357 F.3d 1340, 1348 (Fed. Cir.), *cert. denied*, 125 S.Ct. 61 (2004); *Bell Atlantic Network Serv., Inc. v. Covad Communications Group, Inc.*, 262 F.3d 1258 (Fed. Cir. 2001); *SciMed Life Sys., Inc. v. Advanced Cardiovascular Sys., Inc.*, 242 F.3d 1337 (Fed. Cir. 2001).

to be split up into separate applications (revealing the independence of the multiple inventions).[10]

Such subsidiary principles can thus discipline and guide claim construction toward an understanding of claim language that captures what a skilled artisan would understand to be the invention. A substance-focused, sense-driven, context-dependent approach to understanding claim language can provide systematic guidance to applicants and examiners during patent prosecution and to the public thereafter. As IBM *et al.* recently stated in their *amicus* brief in the *Phillips* case (www.patentlyobviousblog.com/files/IBM.pdf, at 18), "[t]ying the meaning of claim terms to the principle of the patentee's actual invention reduces uncertainty."[11]

The United States's recent brief in the *Phillips* case (*see* www.patentlyobviousblog.com/files/govt_phillips_brief.pdf) discusses a number of these principles. It points out that the PTO, in examining patent applications, focuses on the context provided by the specification. *Id.* at 11 (citing PTO rule, 37 C.F.R. § 1.175(d)(1)). It stresses *Markman*'s emphasis on the "'necessarily sophisticated analysis of the whole [patent] document required by the standard construction rule that a term can be defined only in a way that comports with the instrument as a whole.'" *Id.* at 15 (quoting 517 U.S. at 389). Pointing to the statutory presumption of validity, it endorses the principle of avoiding invalidating constructions where more than one interpretation is reasonable. *Id.* at 16. And it notes with evident approval decisions that construe claims "to encompass a feature that the specification describes as essen-

---

[10] *E.g.*, *Honeywell v. Victor Co. of Japan, Ltd.*, 298 F.3d 1317, 1323-24 (Fed. Cir. 2002); *Rambus*, 318 F.3d at 1095.

[11] That brief notes (at 8, 9) the cacophony within the Federal Circuit, as "the meaning of patent claims is being divorced from the context of the invention itself," with an often-unjustified insistence on a "so-called 'plain meaning[,]'" which "may be entirely divorced from the specification and file history, and thus from the intent of the patentee."

tial to the invention, or that the specification used to distinguish the prior art." *Id.* at 19. Much of the discussion, if not every point, supports establishment of the approach to claim construction we urge.

III. THIS CASE IS A GOOD VEHICLE FOR ESTABLISHING THE PROPER SUBSTANCE-FOCUSED, SENSE-DRIVEN, CONTEXT-DEPENDENT APPROACH TO CLAIM CONSTRUCTION

This case is a good example of what goes wrong when a court conducts claim construction mechanically, with blinkers on that shut out the central question of claim-construction inquiry: does the proposed construction sensibly capture the substance of the advance over prior art that is the invention? Rejection of the majority's claim construction would automatically require setting aside the Federal Circuit's judgment.

A. The majority did not conclude, or cite anything to show, that "inhibitor or activator of a protein" carries the adopted construction as its "precise and well-established meaning in the art." *On-Line Tech.*, 386 F.3d at 1137. Instead, the majority briefly asserted that a positive result in the screening method—a differential effect on the test and control cell lines—*defined* what the test is screening for: any such "substance is, as defined by the claimed method itself, an inhibitor or activator of the protein of interest." Pet. App. 7a. That opening and result-determining step, Housey respectfully suggests, makes no sense.

Inserting the majority's definition into the claim produces a nonsensical tautology. The claim effectively becomes: "A method of determining whether a substance is a substance that has a differential effect on certain test- and control-cell lines, comprising observing whether it has a differential effect on those test- and control-cell lines." That construction makes the opening phrase meaningless surplusage; the claim

becomes "entirely circular," with the phrase "contribut[ing] nothing but meaningless verbiage to the definition of the claimed invention." *Harris*, 114 F.3d at 1152.

The majority's construction contradicts ordinary usage. A method of "screening for" something, an "assay" for something (Pet. App. 2a), starts with an *already defined object of search* and tells investigators when they have likely found it. The pre-assay understanding of the object of search is what motivates investigators to design ways to find it. It would be bizarre, if conceivable at all, for a patent claiming a way of "screening for," "discover[ing]," "identif[ying]," something (Col. 1, ll. 8, 11) to define what is being sought by the results of the method for finding it. The majority's construction is analogous to *defining* "perpetrator" (the person who actually committed a crime) as the person identified in a lineup.

That the majority's construction makes no sense is starkly confirmed by the fact that, under it, the "method of determining" (whether a substance is an inhibitor or activator of a protein) is, by definition, inevitably perfect. Anything with the differential effect necessarily and automatically is, by this construction, an "inhibitor or activator of a protein"; and anything not, not. That inescapable consequence of the majority's definition flatly contradicts the district court's construction of the claim language "method of determining" as meaning ascertaining to a high probability but not with 100% certainty. Pet. App. 30a. The Federal Circuit did not question that construction; the Merck group of respondents in the Federal Circuit did not even challenge it.[12] And it is plainly correct, for, as the district court rightly concluded,

---

[12]Another group did not challenge it on *textual* grounds, but merely argued—incorrectly—that Dr. Housey somehow disclaimed the natural meaning of "determining" while prosecuting his applications, an assertion the district court properly rejected.

"determining" here, as in science generally, provides only a "high degree of probability," not certainty, let alone definitional certainty. The majority's construction misunderstands that basic point.

Nor is the majority's construction made sensible by the two specification statements it cites in its second paragraph explaining that its result-as-definition construction is "logical." Pet. App. 7a-8a. The two statements say that the new method allows investigators to "distinguish[]," to "identif[y]," the looked-for "substances which specifically inhibit or []activate the POI," "[i]nhibitors or activators." *See id.* These are statements about how to discover, or find, something, not what defines it.

The majority's construction thus makes no sense, and the two paragraphs set forth to justify that construction do not in fact do so. Moreover, counting against that construction is the consequence that it renders the patents invalid, thus overturning the presumptively correct decision of the PTO to issue the patents. The majority gave no weight to the avoid-needless-invalidity principle in its claim-construction analysis. Specifically, there is no sound reason why the principle is inapplicable when the proponents of the invalidating construction have actually stipulated that invalidity results.

B. The rest of the majority opinion rejects Housey's construction of the claim phrase, "inhibitor or activator of a protein"—a substance that specifically attaches (binds) to the protein molecule (which may be a multi-part complex in which some subunit proteins sometimes reside) to prevent or allow its functioning in intracellular processes. Contrary to the majority, Housey's construction makes sense of the claim language, patent specification, and prosecution history—far better sense as an understanding of the protected invention than the majority's construction.

The majority acknowledged dictionary definitions that *do* require binding for "inhibitor" or "activator" as one standard meaning for the terms as applied to enzymes, one kind of protein. Pet. App. 9a. But the majority never even asked the question whether this meaning was a *permissible* one as applied to proteins more generally. Instead, it concluded only that this was not "the plain meaning" of the phrase, which is the wrong question. *Id.* And the majority suggested no sensible reason that the concededly available "binding" meaning for enzymes would not be available for "protein" in these patents. Indeed, Dr. Housey expressly claimed his method where "the protein is an enzyme" ('281 Patent, Claim 8), and the protein PKC, which is the focus of most of the specification, is identified in the patents themselves as an enzyme (Col. 6, ll. 47-49). If "inhibitor/activator" even for an *enzyme*—a protein defined by its *activity*—sometimes is limited to a particular method of modulating the activity (by binding), there is no good reason the same meaning would be unavailable for "protein" generally.

Expert evidence about how the claims would be understood undermines the majority's rejection of Housey's construction. Harvard Professor Dr. Griffin, a Housey expert, testified that the patent claims, read in light of the specification and with an understanding of the "improvement over the prior art," claim a method of finding substances that modulate the cellular functioning of the target protein *by* directly interacting with (*i.e.*, binding to) it. Declaration of Dr. Griffin dated June 7, 2002 at 1-3. Even Bayer's expert Dr. Fields, who was adversarial to Housey, testified that he understood that (a) the patent claims claim a method of discovering specific inhibitors or activators of a protein, (b) which means substances that modulate the effect of the protein by binding to it.[13]

---

[13] Bayer expert Fields testified that "one aspect of being a specific activator or inhibitor is that it binds directly to that protein of interest." Fields Depo. dated July 2, 2002, at 91 (quoted at Pet. App. 21a). In the

The specification compellingly supports Housey's construction and undermines the majority's. Simply, the reading given by the majority destroys the distinction that the specification repeatedly says is central to the patented method: within the larger class of all substances that alter a particular cellular property, the differential effect on the test and control lines allows the investigator to separate those which achieve the property-altering effect by binding with the protein molecule from those substances which change the property by some *other* mechanism—by interacting with other proteins within cellular metabolic pathways. As quoted at pages 3-6, *supra*, that distinction is made not only in specification passages quoted by the majority, but in passages describing the limitations of prior-art methods and the advance represented by the new method, which is "absolutely specific" for a given protein (Col. 2, ll. 29-32). *E.g.*, Col. 3, ll. 5-10 ("Substances which specifically inhibit or [ ]activate the POI may be distinguished from substances which affect cell morphology or growth *by other mechanisms* . . . ."); Col. 6, ll. 18-20 ("*we distinguish inhibitors or activators of the POI from agents that act upon other cell metabolites to effect a phenotypic change*"). (Emphases added.) Housey's construction captures that distinction, which is at the heart of the invention, whereas the majority's obliterates it.

Nothing else in the specification can support overriding the key distinction. The only passage that even arguably could be read in isolation to suggest as much (though it does

---

same deposition—in an excerpt submitted to the district court in the Bayer part of the claim-construction proceeding—Fields also testified that "it's my understanding that what Housey means *in this claim* is that this assay is one in which one can identify compounds that are specific inhibitors of or activators of the protein of interest, and that's the title." *Id.* at 48-49 (emphasis added). Fields thus made clear that specificity was part of his understanding of the *claim* (and *title* of the patent)—even without the word "specific" in the language—and that specificity entails binding.

nothing to support the majority's construction)[14] is the statement that properly created cell lines will "be sensitive in their growth properties to chemical agents which are capable of binding to, or modifying the biological effects of, the POI." Col. 6, ll. 30-33. But the ", or modifying" phrase cannot *reasonably* be read as disjunctive, as indicating that the claimed method covers *any* substance that modifies the phenotypic characteristic, even without binding to the POI. If the "or" is not just an imprecision ("or" where "and" belongs), the phrase can only sensibly be read as explanatory ("or, more precisely, modifying the effects of by binding to").

The phrase appears in a paragraph that is introduced "Thus" to illustrate the very sentence just quoted (Col. 6, ll. 18-20) that specifically *distinguishes* the substances identified by this method from "agents that act upon other cell metabolites to effect a phenotypic change." It is senseless to read the ", or modifying" phrase to repudiate that distinction, when it is introduced as exemplifying the distinction. Moreover, a few paragraphs later, the specification uses the same grammatical construction: "bind to, or are activated by, the phorbol ester tumor promoters." Col. 7, ll. 17-18. It is undeniable that, in this passage, the activating is *by binding*, not an alternative to binding. *See* Col. 14, ll. 18-63 (phorbol ester binding to PKC). The same grammatical construction just a few paragraphs earlier is naturally understood the same way, "modifying" *by binding*, not as suddenly and irrationally equating what the patents make a great effort to distinguish.

The prosecution history confirms rather than undermines Dr. Housey's repeated explanation of the essence of his

---

[14] The majority also referred (Pet. App. 10a) to two observations in the specification (Col. 6, ll. 37-42) that address the *degree* and *timing* of the inhibitor/activator's effect on the POI's activity. Those passages are irrelevant here, because they do not even purport to address the *mechanism* by which that effect is achieved (binding or something else), which is the point in dispute—as the majority elsewhere noted (Pet. App. 12a).

invention—a new cellular assay that can pick out the specific protein-targeting substances from the larger class of substances that can alter cellular functioning by other mechanisms (with all their potential harmful unwanted side-effects of such nonspecificity). The majority mistakenly read Dr. Housey's statement, in the prosecution history, that certain prior art did not "provide the target protein/ biochemical pathway specificity of the present invention." Pet. App. 11a. That statement simply does not say that the present invention involves any substance that is specific to *either* the target protein *or* a biochemical pathway. The slash (/) does not assert alternatives, but means action specific to the protein's functioning *in* a particular pathway. Indeed, the very passage quoted by the majority says that the new assay "'provides a *specificity between a target protein and its attendant biochemical pathways.*'" *Id.* (emphasis added).

The majority also overread Dr. Housey's prosecution-history statement that inhibitors or activators "'will be found to interact directly with the target protein itself and/or with other proteins in which the target protein also interacts in the form of dimeric or multimeric complexes.'" *Id.* Even by its terms, this statement does not allow interaction with *any* cell component that produces the cell-property difference (the majority's construction); it refers only to interaction with "dimeric or multimeric complexes." This is a narrow, familiar category of proteins consisting of already-conjoined complexes in which catalytic and regulatory subunit proteins often reside (as with the featured PKAc (Col. 7, ll. 32-33); *see* Alberts, *supra*, at 857), so the searched-for inhibitor/activator can interact with the complex (itself a POI) by interacting with any of its subunits. This is hardly a retraction of the key distinction made not only in the specification, but in the very document containing the quoted passage—which distinguishes the prior art being discussed precisely on the ground that its method is *not* limited to "substances which directly interact with the target protein." Declaration of Gerard M.

Housey dated Oct. 22, 1996 (¶¶ 15, 16, 19(a)). The document thus reinforces, rather than undermines, the critical distinction overriden by the majority's construction.

Dr. Housey emphasized this distinction in responding to an examiner rejection of certain claims as anticipated by a patent issued to Sonnenschein. Amendment dated May 26, 1994. Dr. Housey distinguished Sonnenschein on the ground that it used broad definitions of "estrogens" and "anti-estrogens" so that "any substance which exhibits the properties embodied in these definitions, *whether or not it activates or inihibits an estrogen receptor protein*, will be categorized as an estrogen agonist [activator] or antagonist [inhibitor]." *Id.* at 5 (emphasis added). The difference was between protein interaction specifically and "activity" modulation generally: "It is important to note the difference between an estrogen *receptor* agonist or an estrogen *receptor* antagonist as compared to a substance which exhibits estrogenic or anti-estrogenic activity as defined by the inventors. *The latter may interact with some other protein or non-protein molecule. Thus, the methodology of the [Sonnenschein] reference provides no target protein specificity, which is the essential point of the present invention.* " *Id.* at 5-6 (last emphasis added).

****

The patent-invalidating claim construction adopted in this case should not be allowed to stand. It is incorrect, and the case is a good vehicle for providing needed review of claim-construction principles. The Court should grant review now if respondents insist that the en banc Federal Circuit in *Phillips* will assuredly do nothing to affect the ruling in this case, for that can happen only if *Phillips* does not establish proper claim-construction principles. Otherwise, the Court may wish to hold the petition pending the *Phillips* decision. Once *Phillips* is decided, the Court can readily assess whether it establishes a "reasonable probability" of a different analysis

in this case, justifying a GVR here (*Lawrence v. Chater*, 516 U.S. 163, 167 (1996)), or whether claim-construction principles should be reviewed in this case or in *Phillips* itself. Regardless, the patent-defeating ruling in this case, resting on a fundamentally faulty analysis, should not be permitted to become final.[15]

CONCLUSION

The petition for a writ of certiorari should be granted.

Respectfully submitted,

R. TERRANCE RADER
JAMES F. KAMP
RADER FISHMAN & GRAUER PLLC
39533 Woodward Ave.
Suite 140
Bloomfield Hills, MI 48304
(248) 594-0600

RICHARD G. TARANTO
(Counsel of Record)
FARR & TARANTO
1220 19th Street, NW
Washington, DC 20036
(202) 775-0184

November 22, 2004

[15] The present petition is unlike recent petitions seeking to *broaden* sensibly context-specific decisions of the Federal Circuit. *Multi-Tech Systems, Inc. v. Microsoft Corp.*, 125 S. Ct. 61 (2004) (No. 03-1719); *Gaus v. Conair Corp.*, 125 S. Ct. 346 (2004) (No. 04-196). It is also unlike the unsuccessful petition (125 S. Ct. 216 (2004) (No. 04-32)) from the decision in *Liebel-Flarsheim*, which was interlocutory (allowing *Phillips* to be applied at later stages of the case) and which did undertake the critical contextual analysis (relying on claim differentiation and prosecution-history broadening). The present petition challenges a final Federal Circuit decision that mistakenly blinds itself to context and thus adopts too broad a construction.

APPENDIX A

UNITED STATES COURT OF APPEALS
FEDERAL CIRCUIT

Nos. 03-1193, -1210

HOUSEY PHARMACEUTICALS, INC.,
Plaintiff-Appellant,
v.

ASTRAZENECA UK LTD., ASTRAZENECA LP, ASTRAZENECA PHARMACEUTICALS LP, AND AVENTIS PHARMACEUTICALS, INC.,
Defendants-Cross Appellants,

and

BRISTOL-MYERS SQUIBB COMPANY,
Defendant-Cross Appellant,

and

MERCK & CO., INC.,
Defendant-Cross Appellant,

and

ROCHE HOLDINGS, INC., HOFFMANN-LA ROCHE INC., ROCHE LABORATORIES, INC., AND SYNTEX (U.S.A.) INC.,
Defendants,
and WYETH AND WYETH PHARMACEUTICALS, INC.,
Defendants-Cross Appellants.

[May 7, 2004]

Before MAYER, *Chief Judge*, NEWMAN and CLEVENGER, *Circuit Judges*. Opinion for the court filed by Circuit Judge CLEVENGER. Dissenting opinion filed by Circuit Judge PAULINE NEWMAN.

CLEVENGER, *Circuit Judge.*

Housey Pharmaceuticals, Inc. ("Housey") sued Astrazeneca UK Ltd., Astrazeneca LP, Astrazeneca Pharmaceuticals LP, Aventis Pharmaceuticals, Inc., Bristol-Meyers Squibb Co., Merck & Co., Inc., Roche Holdings, Inc., Hoffmann-La Roche Inc., Roche Laboratories, Inc., Syntex (U.S.A.) Inc., Wyeth, and Wyeth Pharmaceuticals, Inc. (collectively "defendants") in the United States District Court for the District of Delaware, alleging infringement of four patents addressing methods of screening for protein inhibitors and activators. The district court construed several of the limitations of the patent claims, including "inhibitor or activator of a protein." *Bayer Ag v. Housey Pharm., Inc.,* No. 01-148-SLR, 2002 WL 31833205 (D.Del. Nov. 12, 2002).[1] Housey subsequently stipulated that, if this construction were not reversed or modified on appeal, its patents would be invalid and not infringed. *Housey Pharm., Inc. v. Abbott Pharm., Inc.,* No. 01-401-SLR (D.Del. Nov. 26, 2002). The district court ordered a final judgment of invalidity and noninfringement. *Housey Pharm., Inc. v. Abbott Pharm., Inc.,* No. 01-401 SLR (D.Del. Nov. 27, 2002). We conclude that the district court's construction of "inhibitor or activator of a protein" was not erroneous, and we therefore affirm the judgment.

I

Housey is the owner by assignment of U.S. Patents No. 4,980,281, No. 5,266,464, No. 5,688,655, and No. 5,877,007, all of which are titled "Method of Screening for Protein Inhibitors or Activators." Concerning the limitation "inhibitor or activator of a protein," the parties treat the use of the

---

[1] The order containing the district court's constructions of the disputed limitations carries a different caption because the court consolidated oral argument on claim construction in the instant case with a co-pending matter involving the same patents.

limitation in claim 1 of the '281 patent as representative, and we do likewise:

> 1. A method of determining whether a substance is *an inhibitor or activator of a protein* whose production by a cell evokes a responsive change in a phenotypic characteristic other than the level of said protein in said cell per se, which comprises:
>
> (a) providing a first cell line which produces said protein and exhibits said phenotypic response to the protein;
>
> (b) providing a second cell line which produces the protein at a lower level than the first cell line, or does not produce[] the protein at all, and which exhibits said phenotypic response to the protein to a lesser degree or not at all;
>
> (c) incubating the substance with the first and second cell lines; and
>
> (d) comparing the phenotypic response of the first cell line to the substance with the phenotypic response of the second cell line to the substance.

'281 patent, col. 24, ll. 46-63 (emphasis added).

The patented method is an assay, or test, to determine whether a substance, often a candidate for a pharmaceutical product or drug, is an "inhibitor or activator" of a particular protein (the "protein of interest" or "POI") in a cell. Steps (a) and (b) of the method require the existence of two different cell lines. One cell line produces more of the POI, and the other produces less, or none, of the POI. (The cell line producing more of the POI is sometimes referred to as the "test" cell line, and the cell line producing less or none of the POI is sometimes referred to as the "control" cell line.) Additionally, the two cell lines are differentiated by the fact that the increase in the amount of the POI in one of the cell

lines modifies some characteristic in that cell line. In the language of claim 1 of the '281 patent, the relevant characteristic of each of the cell lines is labeled a "phenotypic response" to a different concentration of the POI, and the difference between the relevant characteristic in the two cell lines (presumably caused by the increase in the POI) is a "responsive change in a phenotypic characteristic." In step (c) of the method, the substance being screened is added to each of the cell lines. Step (d) requires comparing the effect that the substance has on the phenotypic response to the POI in each cell line.

In Housey's words, the method requires treating the two cells "with a test substance to determine whether that substance inhibits the cellular functioning of the POI . . . as measured by modulations of the responsive change in the phenotypic characteristic of [one] cell, compared with the modulations of the same responsive phenotypic characteristic, if any, in the [other] cell." The method is allegedly valuable because if the substance being screened impacts the phenotypic response of the cell with an increased concentration of the POI and does not affect the cell containing less or none of the POI in the same way, a logical guess is that the substance has this differential impact because the substance has an effect on the POI. This knowledge is valuable: If a POI is believed to be implicated in a certain disease, and a substance is known to affect that POI, then the substance is more likely to have some value in treating the disease than another substance which does not affect the biological activity of the POI.

II

The district court conducted a *Markman* hearing and construed five claim terms disputed by the parties, including

"inhibitor or activator of a protein,"[2] which was held to mean "a substance that has a greater effect on the phenotype of cells that express the protein of interest at a higher level than on the phenotype of cells that express the protein of interest at a lower level or not at all."

The district court considered and expressly rejected Housey's argument that an "activator or inhibitor of a protein" was limited to substances that "directly interact [with], *i.e.*, bind to, the target protein." The interpretation adopted by the district court includes within an "inhibitor or activator of a protein" substances that affect the test cell's "responsive change in a phenotypic characteristic" through the direct-binding concept to which Housey argues the term is absolutely limited. In addition, however, it is broader: it includes substances that operate through other indirect concepts of molecular interaction. For example, under the district court's interpretation, a substance being screened may still be an "inhibitor or activator of a protein" if it binds to and alters the metabolic functioning of a molecule other than the POI that is essential to either a biochemical pathway required to produce the POI in the first place or a biochemical pathway in which the fully formed POI participates in the cell in order to trigger the "phenotypic response." In other words, the district court's definition includes within the scope of an "inhibitor or activator of a protein" substances that inhibit or activate the biological activity of the POI in the cell without binding to the POI itself.

Housey stipulated that, "based upon the Court's construction of the claim limitation 'inhibitor or activator of a protein,' . . . [and] subject to its right to appeal the Court's

---

[2] The district court's constructions of the other four disputed claim terms are contested either by Housey in its appeal, or by the defendants in a conditional cross-appeal. Because our holding on the construction of "inhibitor or activator of a protein" is dispositive by virtue of Housey's stipulation, we do not address these other terms.

claim construction and any final judgment based thereon, that the Court may enter an order stating that the patents in suit are invalid and not infringed." Housey and the defendants, however, "reserve[d] all of their claims, counterclaims, arguments and defenses" if "the Court's determinations on . . . issues of claim construction [related to 'inhibitor or activator of a protein'] are reversed or modified on appeal such that the matter is remanded for further consideration." Based on the stipulation, the district court entered final judgment in favor of the defendants.

Housey appealed to us, and we have jurisdiction to hear the appeal pursuant to 28 U.S.C. § 1295(a)(1).

III

We review the district court's claim construction de novo. *Cybor Corp. v. FAS Techs., Inc.,* 138 F.3d 1448, 1456 (Fed. Cir.1998) (en banc). Claim construction begins with the language of the claims. *Johnson Worldwide Assocs., Inc. v. Zebco Corp.,* 175 F.3d 985, 989 (Fed. Cir.1999). When construing patent claims, there is a "heavy presumption" that the language in the claim "carries its ordinary and customary meaning," *CCS Fitness, Inc. v. Brunswick Corp.,* 288 F.3d 1359, 1366 (Fed. Cir.2002), "amongst artisans of ordinary skill in the relevant art at the time of the invention," *ResQNet.com, Inc. v. Lansa, Inc.,* 346 F.3d 1374, 1378 (Fed. Cir.2003). Dictionaries and treatises may provide insight into a term's ordinary meaning thus defined. *Tex. Digital Sys., Inc. v. Telegenix, Inc.,* 308 F.3d 1193, 1202-03 (Fed. Cir.2002). However, a claim must also be considered in the context of the intrinsic evidence, namely the claims, the specification, and the prosecution history. *Rexnord Corp. v. Laitram Corp.,* 274 F.3d 1336, 1342-43 (Fed. Cir.2001). If there is a discernable plain and ordinary meaning of the claim language, then this meaning usually defines the scope of the claims unless the patentee has explicitly disclaimed or clearly disavowed this meaning in the specification or prosecution

history. *Liebel-Flarsheim Co. v. Medrad, Inc.*, 358 F.3d 898, 906 (Fed. Cir.2004) (requiring "words or expressions of manifest exclusion or restriction" before broad terms in a claim will be read narrowly in light of a narrow specification (quoting *Teleflex, Inc. v. Ficosa N. Am. Corp.*, 299 F.3d 1313, 1327 (Fed. Cir.2002)); *Middleton, Inc. v. Minn. Mining Mfg. Co.*, 311 F.3d 1384, 1388 (Fed. Cir.2002) ("This court also considers the prosecution history . . . to determine whether the applicant clearly and unambiguously 'disclaimed or disavowed [any interpretation] during prosecution in order to obtain claim allowance.'") (quoting *Standard Oil Co. v. Am. Cyanamid Co.*, 774 F.2d 448, 452 (Fed. Cir.1985) (alteration in original)). For example, the patentee may act as his own lexicographer and expressly define the term in the specification. *See Tex. Digital Sys.*, 308 F.3d at 1204; *Rexnord*, 274 F.3d at 1342.

A

The language of claim 1 of the '281 patent clearly supports the district court's construction and is inimical to any narrower construction. The claim recites "[a] method of determining whether a substance is an inhibitor or activator of a protein." '281 patent, col. 24, ll. 46-47. The steps of the method in turn permit a determination of whether substances affect phenotypic response to the POI in the test and control cells differently. Thus, if "a substance . . . has a greater effect on the phenotype of cells that express the protein of interest at a higher level than on the phenotype of cells that express the protein of interest at a lower level or not at all," the substance is, as defined by the claimed method itself, an inhibitor or activator of the protein of interest.

Furthermore, the specification expressly defines an "inhibitor or activator of a protein" as a substance that yields a positive response to the claimed method, just as the district court did. *See id.* at col. 3, ll. 5-10 ("Substances which specifically inhibit or inactivate [sic] the POI may be dis-

tinguished from substances which affect cell morphology or growth by other mechanisms in that they will have a greater effect on the test lines than on the control line."); *id.* at col. 4, ll. 48-50 ("Inhibitors or activators are identified by their greater effect on the phenotype of the higher producing cell line.").

Housey argues that the district court's construction "confuses the method of distinguishing activators or inhibitors with the definition of activators or inhibitors." We disagree. The claim addresses a method of determining whether a compound is an inhibitor or activator that is conducted by observing whether a particular event occurs when the method is performed. It is therefore logical to construe an "inhibitor or activator of a protein" as a substance that precipitates that event, especially given that the specification, too, defines an inhibitor or activator in the same way.

B

Housey's principal argument on appeal with respect to the construction of "inhibitor or activator of a protein" is the same one that was rejected by the district court: Housey argues that this limitation is "properly construed to mean substances that interact with, *i.e.,* bind to, the POI in order to exert their inhibiting or activating effect on the cellular functioning of the POI." We do not agree that, as used in the '281 patent, an "inhibitor or activator of a protein" is limited to substances that achieve their effect on the biological activity of the POI through a particular reaction mechanism, namely binding to the POI. A substance need only have "a greater effect on the phenotype of cells that express the protein of interest at a higher level than on the phenotype of cells that express the protein of interest at a lower level or not at all" to be an "inhibitor or activator of a protein," and an inhibitor or activator of a POI may alter the biological activity of the POI through a direct POI-binding mechanism or an

indirect pathway-binding mechanism. The word "bind" or "binding" appears nowhere in the '281 patent in conjunction with an "inhibitor or activator of a protein."

At oral argument (and thus on the late side of things), Housey produced definitions of "inhibitor" and "activator" from technical dictionaries to argue for the first time on appeal that the plain meaning of an "inhibitor or activator of a protein" is limited to a substance that binds to the protein. With respect to an inhibitor or activator of an *enzyme,* these definitions do include a direct-binding limitation. *See Chambers Dictionary of Science and Technology* 13 (1999) (defining "activator" as "[o]f an enzyme, a small molecule which binds to it and increases its activity"); *Henderson's Dictionary of Biological Terms* 284 (11th ed.1995) (defining "inhibitor" as "a substance which . . . prevents the normal action of an enzyme . . . by binding to the active site . . . [or] by binding to other parts of the enzyme"). However, the claim language is an "inhibitor or activator of a protein," not of an enzyme,[3] and the dictionaries also include a more appropriate general definition of both "activator" and "inhibitor" that requires only an effect on the biological activity of the protein, regardless of the mechanism used to achieve that effect. *See Chambers Dictionary of Science and Technology* 13 (defining "activator" as "[a]ny agent . . . whose presence stimulates biochemical or physiological changes"); *Henderson's Dictionary of Biological Terms* 284 (defining "inhibitor" as "any agent which checks or prevents an action or process"). Thus, according to the evidence submitted by Housey, the plain meaning of "inhibittor or activator of a protein" does not support the inclusion of the direct-binding concept as a limitation on the claimed method.

---

[3] Enzymes are only one of many types of proteins. *See Henderson's Dictionary of Biological Terms* 474 (noting that proteins are "[e]ssential in living organisms as enzymes, structural constituents of cells and tissues and in control of gene expression etc.").

The intrinsic evidence of the '281 patent does not clearly disavow this broad plain meaning. In fact, even if the plain meaning were considered to be ambiguous, supporting both a narrower definition limited to inhibitors and activators that bind to the POI and a broader definition of inhibitors and activators that need only affect the biological activity of the POI, the district court was correct because the specification and prosecution history affirmatively demonstrate that Housey intended the broader meaning that is not limited to direct binding.

The specification foresaw the possibility of a substance affecting the "responsive change in a phenotypic characteristic" in the cell line with the greater amount of POI without the substance binding to the POI. *See* '281 patent, col. 6, ll. 29-33 ("[C]ell lines will be sensitive in their growth properties [i.e. in the their relevant phenotypic characteristic] to chemical agents which are capable of binding to, *or modifying the biological effects of,* the POI." (emphasis added)).[4] The specification also expressly defines the terms "inhibitors" and "activators" in terms of the biological activity of the POI without any reference to binding. *See id.* at ll. 40-42 ("The term 'inhibitors' includes both substance[s] which reduce the activity of the POI and th[o]se which nullify it altogether."); *id.* at ll. 37-40 ("The term 'activators,' as used herein, includes both substances necessary for the POI to become active in the first place, and substances which merely accentuate its activity.").

Housey's statements in the prosecution history of the '281 patent also support this conclusion. In distinguishing prior art

[4] Housey's argument that the "or" in this passage from the specification is explanatory, not disjunctive, is strained. "Binding to" is unquestionably narrower than "modifying the biological effects of." Rarely does one use "or" in the explanatory sense to define a word as simple as "bind," and even less frequently does one use a broad, general term to explain the meaning of a narrow, precise term.

during prosecution, Housey repeatedly characterized his invention as one that could determine whether a substance interacted with the biological pathway in which a particular protein functioned: The prior art did "not provide the *target protein/biochemical pathway specificity* of the present invention." Because biological pathways usually involve more than one protein, a substance can clearly interact with the POI's biological pathway without binding with the POI itself, and Housey stated as much in the process of distinguishing another prior art reference:

> Substances . . . identified or characterized using the teachings of the Housey patents will be found to interact directly with the target protein itself and/or with other proteins in which the target protein also interacts in the form of dimeric or multimeric complexes. In this fashion, the approach disclosed in the Housey patents provides a *specificity* between a target protein and its attendant biochemical pathways within the cell that is impossible to achieve with other assay systems.

Housey argues that an inhibitor or activator is limited to substances that bind to the POI because the claim language specifies an inhibitor or activator "of a protein." The fact that the biological activity "of a protein" is affected by a screened substance, however, lends no insight into whether the effect must be attributed to binding to the protein or some other type of influence on the biological pathways through which the protein is created and in which the protein functions.

Similarly, Housey argues that the recurring presence of the terms "specific" and "specifically" in the specification and the prosecution history conclusively demonstrates that the inhibitor or activator operates by binding with the POI. *See, e.g.*, '281 patent, col. 1, ll. 10-12 ("In particular, [the invention] is concerned with a method of screening for substances which *specifically* inhibit or activate a particular protein." (emphasis added)); *id.* at col. 2, ll. 27-28 (describing

the invention as having a "specificity for detecting an active agent exceeding that of" the prior art assays); *id.* at ll. 28-32 ("[T]he method which we describe herein involves the generation of a cell line purposefully engineered to detect both stimulatory and inhibitory agents which are absolutely *specific* for any given protein which affects the cultural of morphological characteristics of a cell." (emphasis added)); *id.* at col. 9, ll. 30-31 (stating that the method was screening for "substances which may contain biologically active agents *specific* to the POI" (emphasis added)). Leaving aside the absence of the word "specific" in the claim language (and our repeated warnings not to import limitations from the specification that are not present in the claims, *Amgen Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1325 (Fed. Cir.2003) ("The danger of improperly importing a limitation is even greater when the purported limitation is based upon a term not appearing in the claim.")), this language from the specification does not lead to a strong inference of direct binding to the POI. As the above-quoted sections of the prosecution history demonstrate, the use of "specific" suggests equally as strongly that the biological activity of the POI is affected, through a direct or indirect mechanism, when the biological activity of other proteins in other biochemical pathways is not. Furthermore, the specification, too, equates specificity with the biological effect of the inhibitor or activator, not with any particular mechanism of action. *See* '281 patent, col. 3, ll. 5-10 ("Substances which *specifically* inhibit or inactivate the POI may be distinguished from substances which affect cell morphology or growth by other mechanisms in that they will have a greater effect on the test lines than on the control line." (emphasis added)).[5]

---

[5] Housey's reference to the definition of the term "specific binding" is not relevant to the use of "specific" in the patent, as "specific binding" presupposes the presence of binding, the very limitation which is in question here.

Finally, Housey points to language at the end of one of the specific embodiments in the specification and argues that it demonstrates that the patented assay must be able to detect substances that bind to the POI: "[T]his work establishes, for the first time, the fact that stable overproduction of a protein in mammalian cells can result in a novel cellular phenotype(s) . . . which can be directly modulated by chemical agents *which interact with the protein*." *Id.* at col. 18, ll. 30-36 (emphasis added). First, this passage does not indicate that the interaction between the agent being screened and the POI must be in the form of direct binding; another protein or cellular component may provide the mechanism through which the agent interacts with the POI. Second, not only does this passage describe only a single embodiment (and limitations on specific embodiments are not to be imported into the claims, *see Electro Med. Sys., S.A. v. Cooper Life Scis., Inc.*, 34 F.3d 1048, 1054 (Fed. Cir.1994)), but it describes an embodiment in which there is "a direct relationship between the molar amount of inhibitor [or activator] required to prevent [or stimulate] [the phenotypic response] and the molar amount of [the POI] present in each cell line." '281 patent, col. 18, ll. 22-24, 28-30. This additional fact notes a direct relationship between the amount of POI overexpressed in the test cell and the amount of inhibitor or activator required to eliminate the "responsive change in a phenotypic characteristic." If true, this fact may or may not permit one of skill in the art to use the claimed method to divine that the inhibitor or activator binds directly to the POI, but it unquestionably is not a claimed limitation in the "method of determining whether a substance is an inhibitor or activator of a protein" of claim 1 of the '281 patent.

C

Housey invokes two canons of claim construction to support limiting an "inhibitor or activator of a protein" to a substance that binds to that protein, but neither is persuasive.

First, Housey cites *Athletic Alternatives, Inc. v. Prince Manfacturing, Inc.*, 73 F.3d 1573, 1581 (Fed. Cir.1996), for the proposition that in the event of a tie between a broader and narrower meaning, the notice function of patent law requires that the narrower meaning prevails. Housey misreads *Athletic Alternatives*. In that unusual case, one patent applicant made two *contradictory* and *irreconcilable* affirmative representations of the contested limitation. In those circumstances, we held that the narrower interpretation trumps the broader interpretation. In this case, there are no contradictory and irreconcilable choices for the meaning of "inhibitor or activator of a protein." Second, Housey relies on the axiom that "[c]laims should be so construed, if possible, as to sustain their validity." *Rhine v. Casio, Inc.*, 183 F.3d 1342, 1345 (Fed. Cir.1999) (quoting *Carman Indus., Inc. v. Wahl*, 724 F.2d 932, 937 n. 5 (Fed. Cir.1983)). Here, we have not been asked to review validity, and the district court did not pass on validity. Housey's stipulation of invalidity alone is insufficient to invoke this canon. In any event, the canon lacks dispositive effect in this case. *See Liebel-Flarsheim Co.*, 358 F.3d at 911 ("[U]nless the court concludes, after applying all the available tools of claim construction, that the claim is still ambiguous, the axiom regarding the construction to preserve the validity of the claim does not apply.").

IV

We agree with the district court's construction of the "activator or inhibitor of a protein" limitation, and we therefore affirm the judgment of the district court.

COSTS

No costs.

AFFIRMED.

PAULINE NEWMAN, *Circuit Judge*, dissenting.

It is time to restore the law of claim construction to a more apt wisdom and more usable simplicity. The only issue in this case is the construction of the phrase "inhibitor or activator of a protein." My colleagues' approach to construction is based on confusing recent pronouncements of panels of this court, contravening earlier statements of precedent, thus adding to the confusion.

I

The panel majority propounds the rule that the "plain and ordinary meaning of the claim language" should be used to define the scope of the claims unless the inventor "has explicitly disclaimed or clearly disavowed this meaning." Maj. op. at 1351-1352. The panel majority states that absent such disclaimer, claims are "inimical to any narrower construction." Maj. op. at 1352. Precedent is exactly contrary: a claim is "inimical" to any broader construction than the invention set forth in the specification, and reliance on dictionaries, even technical dictionaries, without due consideration of the context of the invention may lead to "absurd results":

> Indiscriminate reliance on definitions found in dictionaries can often produce absurd results. Words are used in many senses and often have diametrically opposed meanings, depending upon the sense in which they are used. One searching for the dictionary definitions of 'effectively' or 'effect' will find among the numerous definitions listed many which are indisputably inapplicable to the instant use of the word 'effectively.' But the words in which a claim is couched may not be read in a vacuum. One need not arbitrarily pick and choose from the various accepted definitions of a word to decide which meaning was intended as the word is used in a

> given claim. The subject matter, the context, etc., will more often than not lead to the correct conclusion.

Liebscher v. Boothroyd, 46 C.C.P.A. 701, 258 F.2d 948, 951 (1958). *See also e.g., Netword, LLC v. Centraal Corp.*, 242 F.3d 1347, 1352 (Fed. Cir.2001) ("The claims are directed to the invention that is described in the specification; they do not have meaning removed from the context from which they arose."); *Toro Co. v. White Consol. Indus., Inc.*, 199 F.3d 1295, 1299 (Fed. Cir.1999) ("[W]ords of ordinary usage must nonetheless be construed in the context of the patent documents. Thus the court must determine how a person of experience in the field of this invention would, upon reading the patent documents, understand the words used to define the invention."); *Renishaw PLC v. Marposs Societa' per Azioni*, 158 F.3d 1243, 1250 (Fed. Cir.1998) ("[A] common meaning, such as one expressed in a relevant dictionary, that flies in the face of the patent disclosure is undeserving of fealty. . . . [T]he interpretation to be given a term can only be determined and confirmed with a full understanding of what the inventors actually invented and intended to envelop with the claim."). Claims to an invention that is not described in the specification are an anachronism. Many inventions concern complex and detailed technology, and the terse style of the patent claim is not a replacement for the elaboration in the written description. The claims are the concluding portion of the specification, and their statutory function is "particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention." 35 U.S.C. § 112. A claim has no meaning out of the context of the invention that is described, enabled, and prosecuted.

This case again illustrates the conflict generated in the court's recent jurisprudence of claim construction. The panel majority reinforces the recently created dominance of general definitions, wherein the court created a "heavy presumption" in contravention of precedent. It is an established rule of the

construction of legal documents that technical terms are presumed to have the meaning of the technical field of the document, not a "general meaning." Technical terms take their meaning from the technology and context in which they are used, not from general usages of the same word. Thus terms in patent claims are understood in the technical/scientific context of the specification; the presumption is that they have their technical meaning, not a general meaning.

This same interpretive rule applies to statutes, contracts, deeds, patents, and other legal documents. *See, e.g.*, 2A Norman Singer, *Sutherland Statutory Construction* § 47:29 (6th ed. 2000) ("In the absence of legislative intent to the contrary, or other overriding evidence of a different meaning, technical terms or terms of art used in a statute are presumed to have their technical meaning. If a term is connected with and used with reference to a particular trade, the term will have the meaning given [by] experts in the particular trade.") (footnotes omitted).

It is curious indeed to announce a "heavy presumption" that the meaning of a term in a patent claim is unencumbered by the specification, and to place a "heavy burden" on overcoming that presumption. Such a presumption dissociates the term from the invention described, enabled, and prosecuted by the inventor, and places an unnecessary burden on the inventor who wants simply to restore the meaning that the inventor and the examiner, and others in the field of the invention, clearly understand. I thus state my respectful disagreement with the panel's reinforcement of the distortions that have entered this court's jurisprudence. The written description is not an also-ran in claim construction, as the panel states; it is the primary resource in understanding the claims.

Patent claims are stated in words, and words can have a non-technical as well as a technical meaning. The words "inhibit" and "activate" are good examples of such words,

invoking the rule that their specific meaning is derived from the field of their usage, upon inspection of the patent specification and the prosecution history. *See, e.g., Toro*, 199 F.3d at 1299-1300 ("In judicial 'claim construction' the court must achieve the same understanding of the patent, as a document whose meaning and scope have legal consequences, as would a person experienced in the technology of the invention.'"), *further appeal*, 266 F.3d 1367 (Fed. Cir. 2001). The *Toro* court recognized that "dictionary definitions of common words are often less useful than the patent documents themselves in establishing the usage of ordinary words in connection with the claimed subject matter." *Id.* at 1300 (each party relying on a different dictionary definition that favored its position).

The rule is that claims are viewed and understood in light of the specification, of which they are a part. *See, e.g., Multiform Desiccants, Inc. v. Medzam, Ltd.*, 133 F.3d 1473, 1478 (Fed. Cir.1998) ("The best source for understanding a technical term is the specification from which it arose, informed, as needed, by the prosecution history."). If there is a "presumption" in claim construction, it is that a claim term has the meaning ascribed by the patent documents. It is incorrect to impose the obverse "heavy presumption" that the dictionary definition dominates and all inapplicable variants must be expressly disclaimed. The role of dictionaries and treatises is to educate the non-technical judge in understanding what the inventor and the examiner understood, not to impose a new evidentiary presumption and not to enlarge the patented invention beyond that set forth by the inventor. The words of patent claims have the meaning and scope with which they are used in the specification and the prosecution history.

It is also incorrect to hold that words in claims are broader in meaning than their usage in the specification, simply because a dictionary definition may not contain the limi-

tations of the specification. It is imprecise to state that claims are not limited by "a narrow specification" unless the inventor expressly so limits them, maj. op. at 1352, for the specification always limits the claims. The claims are part of the specification; their role is to point out what is covered by the patent, not to broaden the text. *See, e.g., Dow Chemical Co. v. Sumitomo Chemical Co.*, 257 F.3d 1364, 1378 (Fed. Cir.2001) (claim scope is limited to the meaning set forth in the patent documents).

The panel thus perpetuates a distortion of the construction of patent documents, contrary to precedent and defeasible of the critical need for stability and predictability in the system of patents.

II

This case turns on whether the claim clause "inhibitor or activator of a protein" requires direct interaction with the protein. My colleagues on this panel rely on a general understanding of the word "specificity" to decide that the claims cover all forms of indirect interactions and are therefore invalid. The specification, on its face, can support either theory, but does not support the majority's ruling that Housey's proposed limitation of the term "inhibitor or activator" is "inimical" to the majority's proposed "plain meaning" taken from general dictionaries. It is noteworthy that the majority's proposed "plain meaning" differs from the specification's statement of the invention.

The patent documents state that the invention concerns "direct modulation," and that the prior art shows primarily "indirect modulation" of intermediate agents. The defendants dispute this statement. The patentee buttresses its position with selections from the specification, for example:

> Thus, this work establishes, for the first time, the fact that stable overproduction of a protein in mammalian cells can result in a novel cellular phenotype(s) . . .

> which can be directly modulated by chemical agents which interact with the protein.

'281 patent, col. 18, lines 30-36:

> In particular, it is concerned with a method of screening for substances which specifically inhibit or activate a particular protein, especially in a manner apparent to the naked eye.

'281 patent, col. 1, lines 10-12. The defendants cite other, broader statements.

Both sides cite dictionaries and technical treatises that support their respective positions, Housey citing the *Textbook of Receptor Pharmacology* 123 (John C. Foreman & Torben Johansen eds., 1996) for its statement that:

> An all-important consideration in binding studies is the extent to which the measured binding of a radioligand represents association with the receptor or other site of interest. In functional studies there is no difficulty, the response can only be elicited by the binding of an agonist [activator] to the receptor and for an antagonist [inhibitor] to inhibit the response to an agonist it must also bind to the receptor (or a site coupled to it). Invariably, in binding studies there is uptake of the radioligand by other tissue components (unless, of course, binding to a purified, soluble protein is under investigation). The binding to the receptor is normally termed specific binding, whereas the binding to non-receptor tissue components is referred to as nonspecific binding.

The prosecution of the '007 patent referred to the specificity of direct binding; for example, Dr. Housey told the examiner:

> Substances so identified or characterized using the teachings of the Housey patents will be found to interact

> directly with the target protein itself and/or with other proteins which the target protein also interacts in the form of dimeric or multimeric complexes. In this fashion, the approach disclosed in the Housey patents provides a specificity between a target protein and its attendant biochemical pathways within the cell that is impossible to achieve with other assay systems.

Declaration of Gerard M. Housey under 37 C.F.R. § 1.132 at ¶ 14, October 22, 1996.

In a related proceeding involving these patents, [Housey's] expert Dr. Griffin submitted a declaration to the court stating that the terms "specific" and "specifically" are used to characterize a substance that binds to a target protein. Declaration of Dr. James Griffin at 1, June 7, 2002, *Bayer AG v. Housey Pharms., Inc.*, No. 01-148 (D. Del. filed March 6, 2001). On the other hand, Bayer's expert Dr. Roninson provided evidence during a deposition as follows:

> Q. As a scientist, when you see the term "specific inhibitor," do you not understand that to mean substance is binding to the protein?
> A. No, I don't.

Deposition of Igor B. Roninson at 42, June 19, 2002, *Bayer AG v. Housey Pharms., Inc.*, No. 01-148 (D. Del. filed March 6, 2001). But Bayer's expert Dr. Fields stated that "one aspect of being a specific activator or inhibitor is that it binds directly to that protein of interest. . . ." Deposition of Alan P. Fields at 91, July 2, 2002, *Bayer AG v. Housey Pharms., Inc.*, No. 01-148 (D. Del. filed March 6, 2001). And defendants' expert Dr. Silverstein stated before the district court in this litigation that "[a]lthough . . . [drug discovery] programs may include cell-based assays, for the reasons stated above, they must include cell-free assays in order to determine whether or not there is a direct interaction between the test substance and the protein of interest."

Declaration of Professor Saul J. Silverstein at ¶ 22, August 28, 2002, *Housey Pharms., Inc. v. Astrazeneca UK Ltd.*, No. 01-CV-401 (D.Del. November 27, 2002).

This conflicting expert testimony requires resolution by the court, but such resolution must be consistent with the subject matter that was invented, described and enabled. Reliance on dictionaries does not achieve this purpose. In addition, when it is reasonable to do so, claims should be construed so as to preserve their validity. *See Wang Labs. Inc. v. America Online Inc.*, 197 F.3d 1377, 1382 (Fed. Cir.1999) (claims should be construed, when reasonably possible, to sustain their validity).

On correct guidance of the principles of claim construction, I would remand for resolution of conflicting scientific evidence, and re-determination of the meaning and scope of the correctly construed claim.

APPENDIX B

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

C.A. No. 01-401-SLR

HOUSEY PHARMACEUTICALS, INC.,
Plaintiff,

v.

ABBOTT PHARMACEUTICALS, INC., *et al.*,
Defendants.

FINAL JUDGMENT ORDER

WHEREAS the parties have entered into a Stipulation and Order of Invalidity and Non-Infringement; and

WHEREAS the Court has entered a claim construction order in *Bayer v. Housey Pharmaceuticals,* 01-148 (SLR) dated November 12, 2002 which also applies in this action.

IT IS HEREBY ORDERED this 27th day of November, 2002 that final judgment of invalidity and noninfringement of United States Patent Nos. 4,980,281, 5,266,464, 5,688,655 and 5,877,007 ("the patents in suit") is hereby entered in favor of the stipulating defendants[6] in accordance with the terms of the Stipulation and Order of Invalidity and Non-Infringement.

/s/ Sue L. Robinson
United States District Judge

---

[6] AstraZeneca UK Ltd., AstraZeneca LP, AstraZeneca Pharmaceuticals LP, Aventis Pharmaceuticals, Inc., Bristol-Myers Squibb Company, Merck & Co., Inc., Roche Holdings, Inc., Hoffmann-La Roche Inc., Roche Laboratories, Inc., Syntex (U.S.A.) Inc., Wyeth and Wyeth Pharmaceuticals, Inc.

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

C.A. No. 01-401-SLR

HOUSEY PHARMACEUTICALS, INC.,
Plaintiff,

v.

ABBOTT PHARMACEUTICALS, INC., *et al.*,
Defendants.

STIPULATION AND ORDER OF INVALIDITY AND NON-INFRINGEMENT

Plaintiff, Housey Pharmaceuticals, Inc. ("Housey"), and AstraZeneca UK Ltd., AstraZeneca LP, AstraZeneca Pharmaceuticals LP, Aventis Pharmaceuticals, Inc., Bristol-Myers Squibb Company, Merck & Co., Inc., Roche Holdings, Inc., Hoffmann-La Roche Inc., Roche Laboratories, Inc., Syntex (U.S.A.) Inc., Wyeth and Wyeth Pharmaceuticals, Inc., (collectively, the "Defendants") by their undersigned counsel, hereby stipulate and agree, subject to the approval of the Court as follows:

1. This is a patent infringement suit brought by Housey in which Housey claims that Defendants have infringed the claims of U.S. Patent Nos. 4,980,281, 5,266,464, 5,688,655 and 5,877,007 ("the patents in suit").

2. Defendants have counterclaimed that, *inter alia*, the patents in suit are invalid, unenforceable, and not infringed.

3. On November 12, 2002, the Court entered a Memorandum Order regarding claim construction in the co-pending matter styled *Bayer AG v. Housey Pharmaceuticals, Inc.*, C.A. No. 01-148 SLR, attached hereto as Exhibit A.

4. For purposes of judicial economy and based upon the Court's construction of the claim limitation "inhibitor or activator of a protein," Housey hereby stipulates, subject to its right to appeal the Court's claim construction and any final judgment based thereon, that the Court may enter an order stating that the patents in suit are invalid and not infringed.

5. For purposes of judicial economy and based upon the Court's construction of the claim limitation "phenotypic characteristic," Housey hereby stipulates, subject to its right to appeal the Court's claim construction and any final judgment based thereon, that the Court may enter an order stating that the claims of the patents in suit are not infringed by five sets of the defendants (AstraZeneca UK Ltd., AstraZeneca LP, AstraZeneca Pharmaceuticals LP, Aventis Pharmaceuticals, Inc., Bristol-Myers Squibb Company, Merck & Co., Inc., Roche Holdings, Inc., Hoffmann-La Roche Inc., Roche Laboratories, Inc., Syntex (U.S.A.) Inc.).

6. The parties reserve all their rights to argue on appeal that the Court's construction of the other claim limitations should be affirmed, reversed or modified.

7. For purposes of judicial economy, assuming that the Court's Final Judgment Order in this action is affirmed on appeal and the action is not remanded, Defendants will not assert in this action their remaining counterclaims against Housey, specifically, their counterclaims for unenforceability and their counterclaims for attorneys fees.

8. The parties therefore agree to the entry of the attached Final Judgment Order as to both Housey's claim for infringement and Defendants' counterclaims for invalidity and non-infringement. It is provided, however, as to the five sets of defendants identified in

paragraph 5, that: (a) in the event that the Court's determinations on both issues of claim construction set forth in paragraphs 4 and 5 above are reversed or modified on appeal such that the matter is remanded for further consideration, Housey and the five sets of defendants reserve all of their claims, counterclaims, arguments and defenses; and (b) if either of the claim construction issues set forth in paragraphs 4 and 5 above is affirmed on appeal, Housey and the five sets of defendants agree that there is no issue of infringement, validity, or enforceability remaining to be tried. It is further provided, as to defendants Wyeth and Wyeth Pharmaceuticals, Inc., (collectively, "Wyeth"), that: (a) in the event that the Court's determination on the issue of claim construction set forth in paragraph 4 above is reversed or modified on appeal such that the matter is remanded for further consideration, Housey and Wyeth reserve all of their claims, counterclaims, arguments and defenses; and (b) if the claim construction issue set forth in paragraph 4 is affirmed on appeal, Housey and Wyeth agree that there is no issue of infringement, validity, or enforceability remaining to be tried.

9. All declaratory judgment counterclaims are dismissed without prejudice as moot.

BOUCHARD, MARGULES, &
FRIEDLANDER
David J. Margules (#2254)
Joanne P. Pinckney (#3344)
222 Delaware Avenue
Suite 1400
Wilmington, DE 19801
(302) 573-3500
Attorneys for Plaintiff
Housey Pharmaceuticals, Inc.

OF COUNSEL:
Rolf Stadheim
Joseph A. Grear
George C. Summerfield
STADHEIM & GREAR LTD.
400 N. Michigan Avenue
Suite 2200
Chicago, IL 60611-4102
(312) 755-4400

MORRIS, NICHOLS, ARSHT
& TUNNELL
Mary B. Graham (#2556)
Maryellen Noreika (#3208)
1201 N. Market Street
P.O. Box 1347
Wilmington, DE 19899
(302) 658-9200
Attorneys for Defendant
Merck & Co., Inc.

OF COUNSEL
John F. Lynch
Nicolas G. Barzoukas
HOWREY SIMON ARNOLD
& WHITE, LLP
750 Bering Drive
Houston, TX 77057-2198
(713) 787-1400

MORRIS, NICHOLS, ARSHT
& TUNNELL
Jack B. Blumenfeld (#1014)
Karen Jacobs Louden (#2881)
1201 N. Market Street
P.O. Box 1347
Wilmington, DE 19899
(302) 658-9200
Attorneys for Defendants AstraZeneca LP, AstraZeneca Pharmaceuticals LP, Aventis Pharmaceuticals, Inc., Bristol-Myers Squibb Company

OF COUNSEL
Herbert F. Schwartz
Denise L. Loring
FISH & NEAVE
1251 Avenue of the Americas
New York, NY 10020

Robert L. Baechtold
Scott K. Reed
FITZPATRICK CELLA, HARPER
& SCINTO
30 Rockefeller Plaza
New York, NY 10112

POTTER ANDERSON
& CORROON LLP
Philip A. Rovner (#3215)
1313 N. Market Street
P.O. Box 951
Wilmington, DE 19899
(302) 984-6000
Attorneys for Defendant
Roche Holdings, Inc.,
Hoffmann-La Roche Inc.,
Roche Laboratories, Inc.,
and Syntex (USA.), Inc.

OF COUNSEL
ORRICK HERRINGTON & SUTCLIFFE LLP
Brian D. Coggio
Jennifer Gordon
666 Fifth Avenue
New York, NY 10103
(212) 506-5000

YOUNG CONAWAY
STARGATT & TAYLOR
Josy W. Ingersoll (#1088)
1000 West St., 17th Floor
P.O. Box 391
Wilmington, DE 19899
(302) 571-6672
Attorneys for Defendant Wyeth and Wyeth Pharmaceuticals, Inc.

OF COUNSEL
HALE & DORR
William F. Lee
Lisa J. Pirozzolo
60 State Street
Boston, MA 02109

SO ORDERED this ____ day of November, 2002

_____
United States District Judge

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

Civil Action No. 01-148-SLR

BAYER AG and BAYER CORPORATION,
Plaintiffs,

v.

HOUSEY PHARMACEUTICALS, INC.,
Defendant.

MEMORANDUM ORDER

At Wilmington this 12th day of November, 2002, having heard oral argument and having reviewed papers submitted in connection therewith;

IT IS ORDERED that the disputed claim language in United States Patent Nos. 4,980,281; 5,266,464; 5,688,655 and 5,877,077, as identified by the above referenced parties, shall be construed as follows, consistent with the tenets of claim construction set forth by the United States Court of Appeals for the Federal Circuit:

A. "A Method of Determining"

The parties agree that, in science, rarely is anything 100% certain. Consistent with this understanding, the patent specification, and the prosecution history,[7] "determining" shall be construed to mean "ascertaining with a high degree of probability that an unknown or suspected substance is an activator or inhibitor of a target protein."

---

[7] The patentee's 37 C.F.R. § 1.132 declaration states that "the predictive ability of the claimed cell-based system is far higher than that for more traditional cell-free systems." (D.I. 276, Ex. B)

B. "Inhibitor or Activator of a Protein"

The claim language, the specification, and the prosecution history contain no language limiting an activator or inhibitor to substances that directly bind to the target protein. As such, the court rejects the patentee's attempt to read such a limitation into the claims.[8] Thus, the term "inhibitor or activator" shall be construed to mean "a substance that has a greater effect on the phenotype of cells that express the protein of interest at a higher level than on the phenotype of cells that express the protein of interest at a lower level or not at all." ('281 patent, col. 3, 11. 5-10; col. 4, 11. 48-50)

C. "Phenotypic Characteristic"

The term "phenotypic characteristic" shall be construed to mean "an observable trait of a cell and does not include characteristics of a temporary or transient nature, such as, for example, the levels or concentrations of ions or other chemical substances." The patent specification states "[t]he phenotypic characteristic is preferably a 'cultural' or 'morphological' characteristic of the cell." ('281 patent, col. 4, 11. 54-55) Cultural or morphological changes are stable, non-transient traits.

D. "Phenotypic Response" or "Responsive Change in a Phenotypic Characteristic"

The term "phenotypic response" or "responsive change in a phenotypic characteristic" shall be construed to mean "the characteristic which is changing and which the person

---

[8] The patentee has argued that the term should be construed to mean "substances the directly interact, i.e., bind to, the target protein." The court, however, finds no intrinsic evidence to support the direct interaction (binding) limitation. The extrinsic evidence (expert reports) submitted by the patentee are merely conclusory.

skilled in the art is measuring, which is reflective of the activation or inhibition of the protein of interest."[9]

E. "Evokes," "Exhibits," and "Induces"

The term "evokes," "exhibits," and "induces" shall be construed to mean "the phenotypic change in the cell that overproduces the protein of interest is observable prior to the addition of the test substance to that cell." ('464 patent, col 7, 11, 7-15)

/s/ Sue L. Robinson
United States District Judge

[9] The court rejects plaintiffs' attempt to add language regarding reporter genes to the claim construction. Although the "responsive change" must be reflective of the activation or inhibition of the protein of interest, there is no limitation as to how the "responsive change' may be measured. ('655 patent, claim 1)

APPENDIX C

UNITED STATES COURT OF APPEALS
FEDERAL CIRCUIT

Nos. 03-1193, -1210

HOUSEY PHARMACEUTICALS, INC.,
Plaintiff-Appellant,

v.

ASTRAZENECA UK LTD., ASTRAZENECA LP, ASTRAZENECA PHARMACEUTICALS LP, and AVENTIS PHARMACEUTICALS, INC.,
Defendants-Cross Appellants,

and

BRISTOL-MYERS SQUIBB COMPANY,
Defendant-Cross Appellant,

and

MERCK & CO., INC.,
Defendant-Cross Appellant,

and

ROCHE HOLDINGS, INC., HOFFMANN-LA ROCHE INC., ROCHE LABORATORIES, INC., and SYNTEX (U.S.A.) INC.,
Defendants,

and WYETH AND WYETH PHARMACEUTICALS, INC.,
Defendants-Cross Appellants.

ORDER

A combined petition for panel rehearing and for rehearing en banc having been filed by the APPELLANT, and the petition for rehearing having been referred to the panel that

heard the appeal, and thereafter the petition for rehearing en banc having been referred to the circuit judges who are in regular active service,

UPON CONSIDERATION THEREOF, it is

ORDERED that the petition for panel rehearing be, and the same hereby is, DENIED and it is further

ORDERED that the petition for rehearing en banc be, and the same hereby is, DENIED.

The mandate of the court will issue on September 1, 2004.

FOR THE COURT,
Jan Horbaly
Clerk

Dated: August 25, 2004

cc: R. Terrance Rader
W. Lee, J. Lynch, H. Schwartz, R. Baechtold

HOUSEY PHARMACEUTICAL V ASTRAZENECA, 03-1193, -1210 (DCT – 01-CV-401)

(4)

Supreme Court, U.S.
FILED

04-695 NOV 22 2004

OFFICE OF THE CLERK

No. 04-___

IN THE

Supreme Court of the United States

HOUSEY PHARMACEUTICALS, INC.,
Petitioner,

v.

MERCK & CO., INC., ASTRAZENECA UK LTD., ASTRAZENECA LP, ASTRAZENECA PHARMACEUTICALS LP, AVENTIS PHARMACEUTICALS, INC., BRISTOL-MYERS SQUIBB COMPANY, ROCHE HOLDINGS, INC., HOFFMANN-LA ROCHE INC., ROCHE LABORATORIES, INC., SYNTEX (U.S.A.) INC., WYETH, AND WYETH PHARMACEUTICALS INC.,
Respondents.

On Petition for a Writ of Certiorari to the United States Court of Appeals for the Federal Circuit

PATENT APPENDIX

R. TERRANCE RADER
JAMES F. KAMP
RADER, FISHMAN & GRAUER PLLC
39533 Woodward Ave.
Suite 140
Bloomfield Hills, MI 48304
(248) 594-0600

RICHARD G. TARANTO
(Counsel of Record)
FARR & TARANTO
1220 19th Street, NW
Washington, DC 20036
(202) 775-0184

WILSON-EPES PRINTING CO., INC. – (202) 789-0096 – WASHINGTON, D. C. 20001

TABLE OF CONTENTS

	Page
U.S. Patent No. 4,980,281	1b
U.S. Patent No. 5,266,464	36b
U.S. Patent No. 5,688,655	75b
U.S. Patent No. 5,877,007	113b

United States Patent [19]

Housey

[11] **Patent Number: 4,980,281**

[45] **Date of Patent: Dec. 25, 1990**

[54] **METHOD OF SCREENING FOR PROTEIN INHIBITORS AND ACTIVATORS**

[76] Inventor: Gerard M. Housey, 100 Haven Ave., Apt 7E, New York, N.Y. 10032

[21] Appl. No.: 154,206

[22] Filed: Feb. 10, 1988

[51] Int. Cl.[5] C12Q 1/02

[52] U.S. Cl. 435/29; 435/240.1; 435/240.2

[58] Field of Search 435/29

[56] References Cited

U.S. PATENT DOCUMENTS

4,480,038	10/1984	Cheng	435/29
4,532,204	7/1985	Crespi	435/29

OTHER PUBLICATIONS

Housey et al., Cell, vol. 52, 343–354, Feb. 12, 1988.

Julius, et al., Ectopic Expression of The Serotonin 1c Receptor and the Triggering of Malignant Transformation (1989) Science, vol. 244, pp. 1057–1062.

Primary Examiner—Sam Rosen

Attorney, Agent, or Firm—Browdy & Neimark

[57] ABSTRACT

Inhibitors and activators of a protein whose expression affects a phenotypic characteristic of the cell, especially a cultural or morphological characteristic, are identified by their more pronounced effect on cells producing higher, usually non-naturally occurring, levels of the protein, than on cells producing little or none of the protein. In a preferred assay, the effect is observable with the naked eye.

By this method, tamoxifen is identified as an inhibitor of PKC activity in cell culture.

24 Claims, 21 Drawing Sheets

FIG. 1.

```
          10          20          30          40          50          60
          *           *           *           *           *           *
GAA TTC CGC CTC TCC GGG CTT ACA GCC CGC GGT CCC GCC GCC CCG GGG CCG CCA CCT CTC

          70          80          90          100         110         120
          *           *           *           *           *           *
GGG GCT CCC CCC AGT CCC CGC GCG CGC AAG ATG GCT GAC CCG GCT GCG GGG CCG CCG CCG
                                    Met Ala Asp Pro Ala Ala Gly Pro Pro Pro

        130         140         150         160         170         180
          *           *           *           *           *           *
AGC GAG GGC GAG GAG AGC ACG GTG CGC TTC GCC CGC AAA GGG CCC CTC CGG CAG AAG AAC
Ser Glu Gly Glu Glu Ser Thr Val Arg Phe Ala Arg Lys Gly Pro Leu Arg Gln Lys Asn
```

FIG. 1 CONT.

```
      190         200         210         220         230         240
       *           *           *           *           *           *
GTG CAC GAG GTG AAG AAC CAC AAA TTC ACC GCC CGC TTC TTC AAG CAG CCC ACC TTC TGC
Val His Glu Val Lys Asn His Lys Phe Thr Ala Arg Phe Phe Lys Gln Pro Thr Phe Cys

      250         260         270         280         290         300
       *           *           *           *           *           *
AGC CAC TGC ACC GAC TTC ATT TGG GGC TTC GGG AAG CAG GGA TTC CAG TGT CAA GTC TGC
Ser His Cys Thr Asp Phe Ile Trp Gly Phe Gly Lys Gln Gly Phe Gln Cys Gln Val Cys

      310         320         330         340         350         360
       *           *           *           *           *           *
TGC TTT GTT GTA CAC AAG CGC TGC CAT GAA TTC GTC ACG TTC TCC TGC CCT GGT GCA GAC
Cys Phe Val Val His Lys Arg Cys His Glu Phe Val Thr Phe Ser Cys Pro Gly Ala Asp
```

FIG. 1 CONT.

```
          370         380         390         400         410         420
           *           *           *           *           *           *
AAG GGC CCG GCC TCT GAT GAC CCA CGG AGC AAA CAC AAG TTT AAG ATC CAC ACC TAC TCC
Lys Gly Pro Ala Ser Asp Asp Pro Arg Ser Lys His Lys Phe Lys Ile His Thr Tyr Ser

          430         440         450         460         470         480
           *           *           *           *           *           *
AGC CCT ACC TTC TGT GAC CAC TGT GGA TCA CTG CTG TAT GGG CTC ATC CAC CAG GGG ATG
Ser Pro Thr Phe Cys Asp His Cys Gly Ser Leu Leu Tyr Gly Leu Ile His Gln Gly Met

          490         500         510         520         530         540
           *           *           *           *           *           *
AAA TGC GAC ACC TGT ATG ATG AAT GTC CAC AAG CGC TGC GTG ATG AAC GTC CCC AGC CTC
Lys Cys Asp Thr Cys Met Met Asn Val His Lys Arg Cys Val Met Asn Val Pro Ser Leu
```

FIG. 1 CONT.

```
          550         560         570         580         590         600
           *           *           *           *           *           *
TGT GGC ACC GAC CAC ACA GAA CGC CGT GGC CGC ATC TAC ATC CAG GCC CAC ATC GAC AGG
Cys Gly Thr Asp His Thr Glu Arg Arg Gly Arg Ile Tyr Ile Gln Ala His Ile Asp Arg

          610         620         630         640         650         660
           *           *           *           *           *           *
GAG GTC CTC ATC GTT GTT GTA AGA GAT GCT AAA AAT CTG GTA CCT ATG GAC CCC AAC GGC
Glu Val Leu Ile Val Val Val Arg Asp Ala Lys Asn Leu Val Pro Met Asp Pro Asn Gly

          670         680         690         700         710         720
           *           *           *           *           *           *
TTG TCA GAT CCC TAC GTA AAA CTG AAA CTG ATC CCT GAT CCC AAA AGT GAG AGC AAG CAG
Leu Ser Asp Pro Tyr Val Lys Leu Lys Leu Ile Pro Asp Pro Lys Ser Glu Ser Lys Gln
```

FIG. 1 CONT.

```
          730         740         750         760         770         780
           *           *           *           *           *           *
AAG ACC AAG ACT ATC AAA TGC TCC CTC AAC CCG GAG TGG AAC GAA ACC TTC AGA TTT CAG
Lys Thr Lys Thr Ile Lys Cys Ser Leu Asn Pro Glu Trp Asn Glu Thr Phe Arg Phe Gln

          790         800         810         820         830         840
           *           *           *           *           *           *
CTG AAG GAA TCA GAC AAA GAC AGA AGA CTG TCC GTA GAG ATC TGG GAT TGG GAC CTG ACC
Leu Lys Glu Ser Asp Lys Asp Arg Arg Leu Ser Val Glu Ile Trp Asp Trp Asp Leu Thr

          850         860         870         880         890         900
           *           *           *           *           *           *
AGC AGG AAT GAC TTC ATG GGA TCT CTG TCG TTT GGG ATT TCA GAA CTA CAG AAA GCC GGA
Ser Arg Asn Asp Phe Met Gly Ser Leu Ser Phe Gly Ile Ser Glu Leu Gln Lys Ala Gly
```

FIG. 1 CONT.

```
          910         920         930         940         950         960
           *           *           *           *           *           *
GTG GAT GGC TGG TTC AAG TTA CTA AGC CAG GAA GAA GGC GAG TAC TTT AAT GTG CCG GTG
Val Asp Gly Trp Phe Lys Leu Leu Ser Gln Glu Glu Gly Glu Tyr Phe Asn Val Pro Val

          970         980         990        1000        1010        1020
           *           *           *           *           *           *
CCG CCG GAA GAA AGC GAG GGC AAT GAA GAG CTG CGG CAG AAG TTT GAG AGA GCC AAG ATT
Pro Pro Glu Glu Ser Glu Gly Asn Glu Glu Leu Arg Gln Lys Phe Glu Arg Ala Lys Ile

         1030        1040        1050        1060        1070        1080
           *           *           *           *           *           *
GGC CAA GGT ACC AAG GCT CCA GAA GAA AAG ACA GCG AAC ACT ATA TCC AAA TTT GAC AAC
Gly Gln Gly Thr Lys Ala Pro Glu Glu Lys Thr Ala Asn Thr Ile Ser Lys Phe Asp Asn
```

FIG. 1 CONT.

```
      1090        1100        1110        1120        1130        1140
        *           *           *           *           *           *
AAT GGC AAC AGG GAC CGG ATG AAA CTG ACC GAT TTT AAC TTC CTG ATG GTG CTG GGG AAA
Asn Gly Asn Arg Asp Arg Met Lys Leu Thr Asp Phe Asn Phe Leu Met Val Leu Gly Lys

      1150        1160        1170        1180        1190        1200
        *           *           *           *           *           *
GGC AGC TTT GGC AAG GTC ATG CTC TCA GAG CGG AAG GGT ACA GAT GAA CTC TAT GCC GTG
Gly Ser Phe Gly Lys Val Met Leu Ser Glu Arg Lys Gly Thr Asp Glu Leu Tyr Ala Val

      1210        1220        1230        1240        1250        1260
        *           *           *           *           *           *
AAG ATC CTG AAG AAA GAT GTG GTG ATC CAA GAT GAC GAT GTG GAG TGC ACA ATG GTG GAG
Lys Ile Leu Lys Lys Asp Val Val Ile Gln Asp Asp Asp Val Glu Cys Thr Met Val Glu
```

FIG. 1 CONT.

```
          1270        1280        1290        1300        1310        1320
            *           *           *           *           *           *
AAG AGG GTG CTG GCC CTG CCT GGG AAG CCC CCA TTC CTG ACT CAG CTC CAT TCC TGC TTC
Lys Arg Val Leu Ala Leu Pro Gly Lys Pro Pro Phe Leu Thr Gln Leu His Ser Cys Phe

          1330        1340        1350        1360        1370        1380
            *           *           *           *           *           *
CAG ACC ATG GAC CGC CTC TAC TTT GTG ATG GAG TAT GTG AAC GGG GGC GAC CTC ATG TAC
Gln Thr Met Asp Arg Leu Tyr Phe Val Met Glu Tyr Val Asn Gly Gly Asp Leu Met Tyr

          1390        1400        1410        1420        1430        1440
            *           *           *           *           *           *
CAC ATC CAA CAA GTT GGC CGT TTC AAG GAG CCC CAT GCT GTA TTT TAC GCT GCA GAG ATT
His Ile Gln Gln Val Gly Arg Phe Lys Glu Pro His Ala Val Phe Tyr Ala Ala Glu Ile
```

FIG. 1 CONT.

```
          1450        1460        1470        1480        1490        1500
            *           *           *           *           *           *
GCC ATC GGT CTT TTC TTC TTG CAG AGC AAG GGC ATC ATT TAC CGT GAC CTG AAA CTT GAC
Ala Ile Gly Leu Phe Phe Leu Gln Ser Lys Gly Ile Ile Tyr Arg Asp Leu Lys Leu Asp

          1510        1520        1530        1540        1550        1560
            *           *           *           *           *           *
AAC GTG ATG CTG GAT TCC GAG GGG CAC ATC AAA ATC GCT GAC TTT GGC ATG TGT AAA GAG
Asn Val Met Leu Asp Ser Glu Gly His Ile Lys Ile Ala Asp Phe Gly Met Cys Lys Glu

          1570        1580        1590        1600        1610        1620
            *           *           *           *           *           *
AAT ATC TGG GAT GGG GTG ACA ACC AAG ACA TTC TGT GGC ACT CCA GAC TAC ATT GCC CCA
Asn Ile Trp Asp Gly Val Thr Thr Lys Thr Phe Cys Gly Thr Pro Asp Tyr Ile Ala Pro
```

FIG. 1 CONT.

```
        1630        1640        1650        1660        1670        1680
         *           *           *           *           *           *
GAG ATC ATT GCT TAT CAG CCC TAC GGA AAG TCT GTG GAC TGG TGG GCG TTT GGA GTC CTG
Glu Ile Ile Ala Tyr Gln Pro Tyr Gly Lys Ser Val Asp Trp Trp Ala Phe Gly Val Leu

        1690        1700        1710        1720        1730        1740
         *           *           *           *           *           *
CTG TAT GAA ATG TTG GCT GGC CAG GCA CCT TTT GAA GGG GAG GAT GAG GAT GAA CTC TTC
Leu Tyr Glu Met Leu Ala Gly Gln Ala Pro Phe Glu Gly Glu Asp Glu Asp Glu Leu Phe

        1750        1760        1770        1780        1790        1800
         *           *           *           *           *           *
CAG TCA ATC ATG GAG CAC AAC GTG GCG TAT CCC AAG TCC ATG TCT AAG GAA GCT GTG GCA
Gln Ser Ile Met Glu His Asn Val Ala Tyr Pro Lys Ser Met Ser Lys Glu Ala Val Ala
```

FIG. 1 CONT.

```
        1810        1820        1830        1840        1850        1860
         *           *           *           *           *           *
ATC TGC AAA GGG CTA ATG ACC AAA CAC CCA GGC AAG CGC CTG GGT TGT GGG CCT GAA GGG
Ile Cys Lys Gly Leu Met Thr Lys His Pro Gly Lys Arg Leu Gly Cys Gly Pro Glu Gly

        1870        1880        1890        1900        1910        1920
         *           *           *           *           *           *
GAA CGA GAC ATT AAG GAG CAT GCA TTT TTC CGG TAT ATC GAC TGG GAG AAA CTC GAA CGC
Glu Arg Asp Ile Lys Glu His Ala Phe Phe Arg Tyr Ile Asp Trp Glu Lys Leu Glu Arg

        1930        1940        1950        1960        1970        1980
         *           *           *           *           *           *
AAG GAG ATT CAG CCA CCT TAT AAA CCA AAA GCT AGA GAC AAG CGA GAC ACC TCC AAC TTC
Lys Glu Ile Gln Pro Pro Tyr Lys Pro Lys Ala Arg Asp Lys Arg Asp Thr Ser Asn Phe
```

FIG. 3.

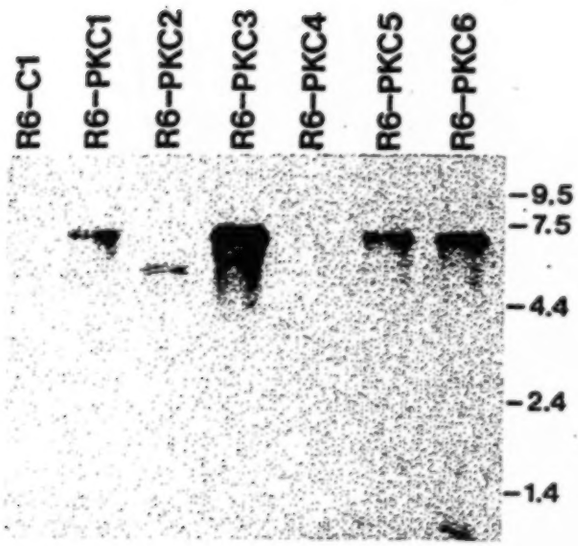

R6-C1
R6-PKC1
R6-PKC2
R6-PKC3
R6-PKC4
R6-PKC5
R6-PKC6
-9.5
-7.5
-4.4
-2.4
-1.4

FIG. 4.

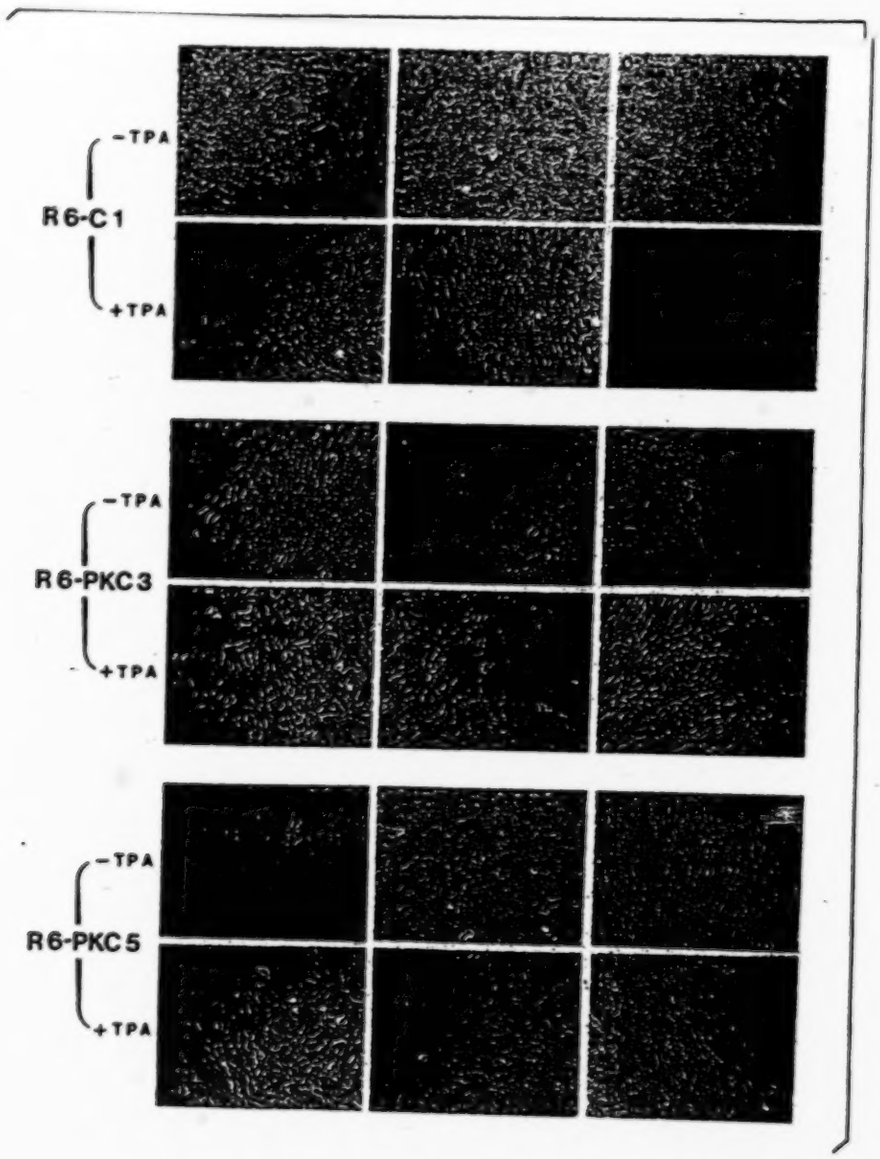

-TPA
R6-C1
+TPA
-TPA
R6-PKC3
+TPA
-TPA
R6-PKC5
+TPA

FIG. 5.

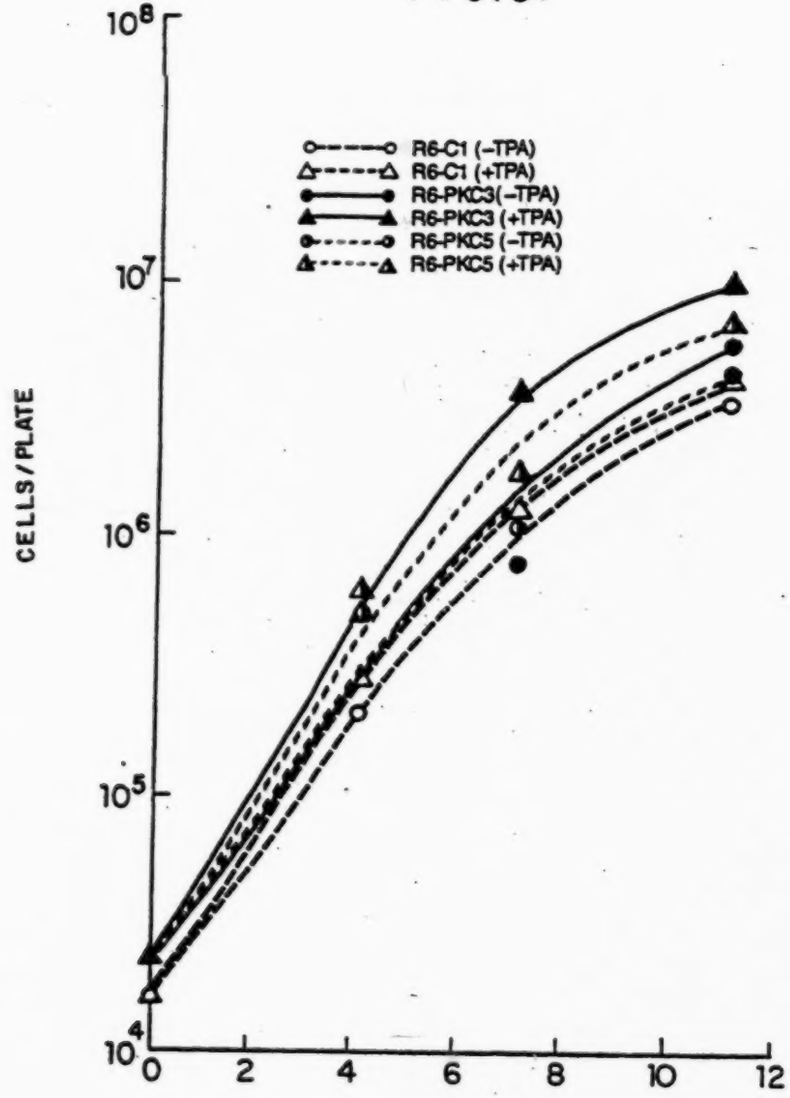

R6-C1 (–TPA)
R6-C1 (+TPA)
R6-PKC3(–TPA)
R6-PKC3 (+TPA)
R6-PKC5 (–TPA)
R6-PKC5 (+TPA)
CELLS/PLATE
10^8
10^7
10^6
10^5
10^4
0
2
4
6
8
10
12
Days

FIG. 6.

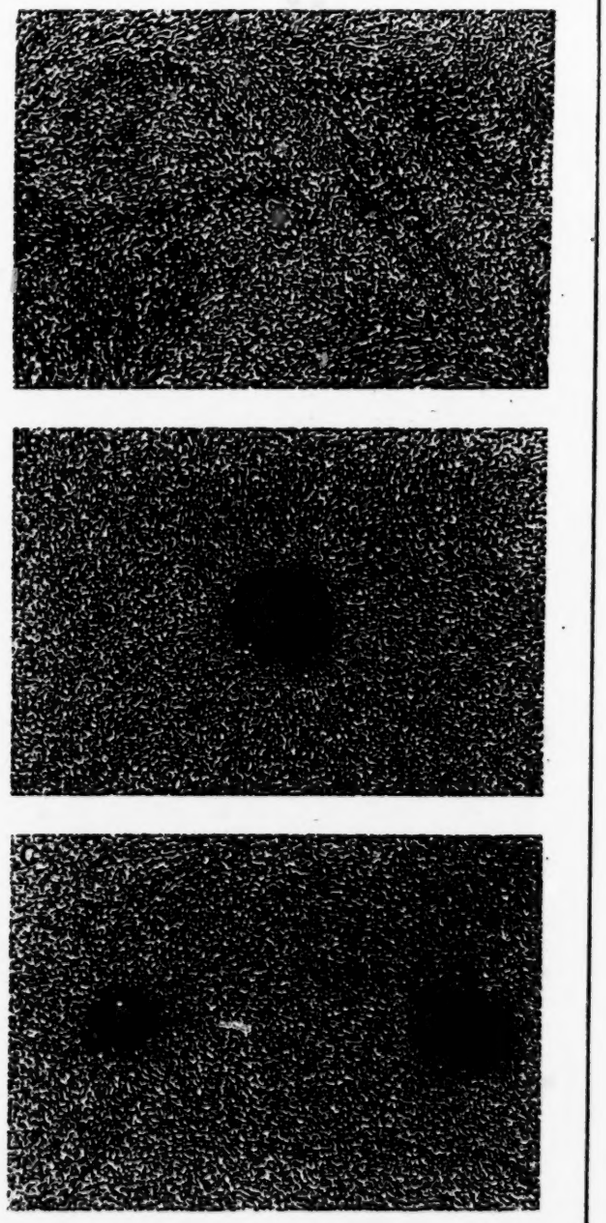


FIG. 1 CONT.

```
        1990        2000        2010        2020        2030        2040
          *           *           *           *           *           *
GAC AAA GAG TTC ACC AGG CAG CCT GTG GAA CTG ACT CCC ACT GAC AAA CTC TTC ATC ATG
Asp Lys Glu Phe Thr Arg Gln Pro Val Glu Leu Thr Pro Thr Asp Lys Leu Phe Ile Met

        2050        2060        2070        2080        2090        2100
          *           *           *           *           *           *
AAC TTG GAC CAA AAT GAA TTT GCT GGC TTC TCG TAT ACT AAC CCA GAG TTT GTC ATT AAT
Asn Leu Asp Gln Asn Glu Phe Ala Gly Phe Ser Tyr Thr Asn Pro Glu Phe Val Ile Asn

        2110        2120        2130        2140        2150        2160
          *           *           *           *           *           *
GTG TAG GTG AAT GCA GAT TCC ATC GCT GAG CCT GTG TGT AAG GCT GCA GCG TGA ATG TCT
Val ---
```

FIG. 1 CONT.

```
        2170        2180        2190        2200        2210        2220
         *           *           *           *           *           *
ATT ATC AAT TCC AGT CTT CCA GGA TTC ATG GTG CCT CTG TTG GCA TCC GTC ATG TGG AGA

        2230        2240        2250        2260        2270        2280
         *           *           *           *           *           *
GCT TGT CTT AGA GGG CTT TTC TTT GTA TGT ATA GCT TGC TAG TTT GTT TTC TAC ATT TCA

        2290        2300        2310        2320        2330        2340
         *           *           *           *           *           *
AAA TGT TTA GTT TAG AAT AAG TGC ATT GCC CAC TGA TAG AGG TAC AAT TTT CCA GAC TTC
```

FIG. 1 CONT.

```
      2350        2360        2370        2380        2390        2400
       *           *           *           *           *           *
CAG AAA CTC ATC CAA TGA ACC AAC AGT GTC AAA ACT TAA CTG TGT CCG ATA CCA AAA TGC

      2410        2420        2430        2440        2450        2460
       *           *           *           *           *           *
TTC AGT ATT TGT AAT TTT TAA AGT CAG ATG CTG ATG TTC CTG GTC AAA GTT TTT ACA GTT

      2470        2480        2490        2500        2510        2520
       *           *           *           *           *           *
ACT CTC GAA TAT CTC CTT TGA ATG CTA CCT AAG CAT GAC CGG TAT TTT TAA AAG TTG TGA
```

FIG. 1 CONT.

```
          2530        2540        2550        2560        2570        2580
            *           *           *           *           *           *
GTA AGC TTT GCA GTT ACT GTG AAC TCT TGT CTC TTG GAG GAA CTT TTT GTT TAA GAA TTG

          2590        2600
            *           *
GTA TGA TTA AAC TGA ATT C*
```

BEST AVAILABLE COPY

FIG. 2.

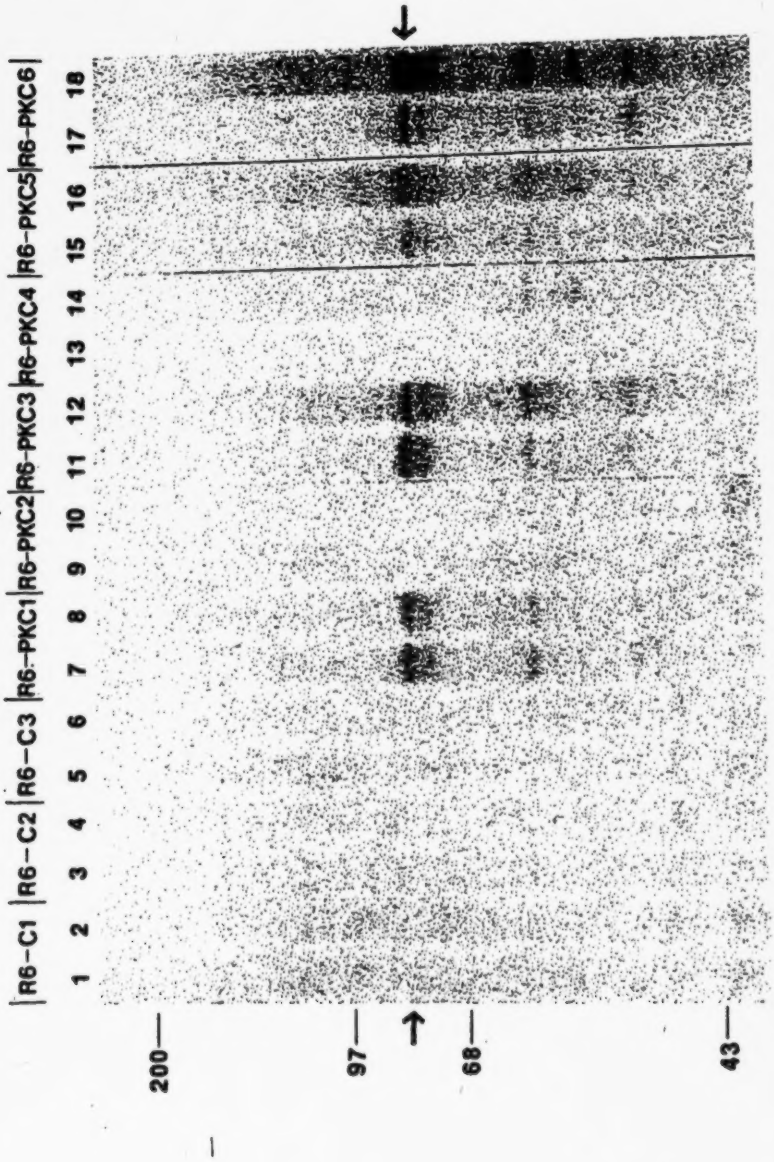
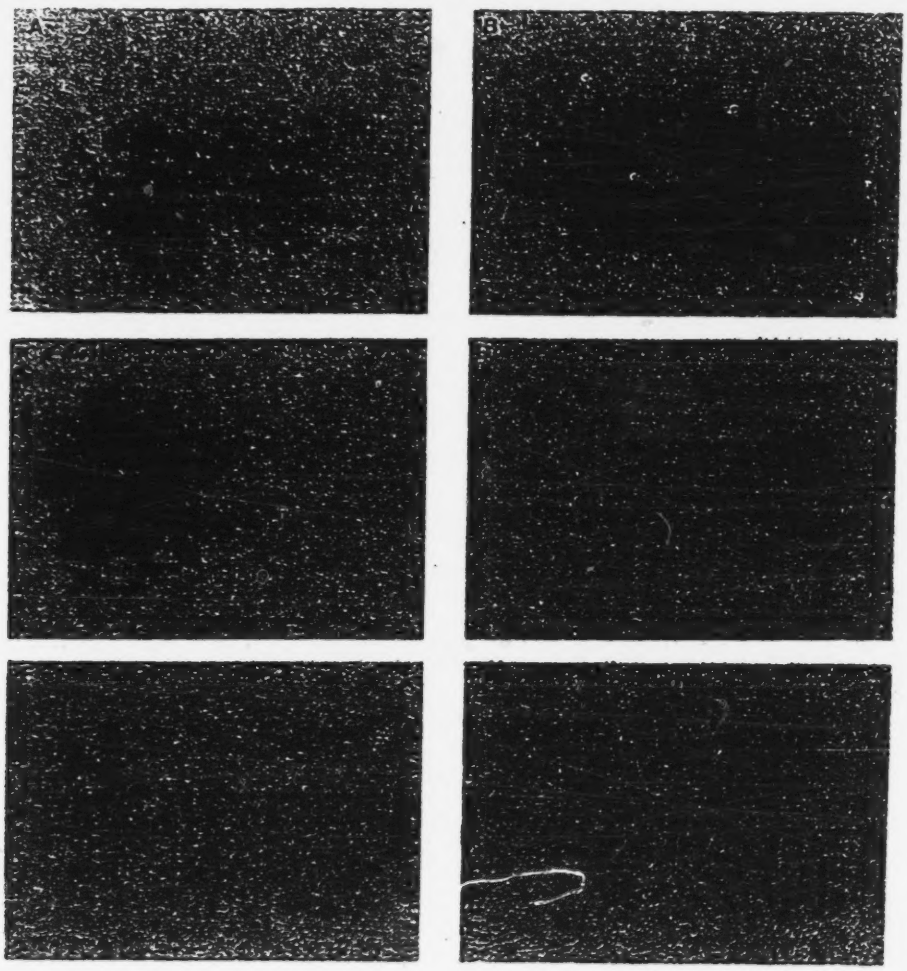


FIG. 7.



METHOD OF SCREENING FOR PROTEIN INHIBITORS AND ACTIVATORS

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a general screening method for the discovery and identification of both inhibitors and activators of enzymes, receptors, and other proteins. In particular, it is concerned with a method of screening for substances which specifically inhibit or activate a particular protein affecting the cultural or morphological characteristics of the cell expressing the protein, especially in a manner apparent to the naked eye.

2. Information Disclosure Statement

A number of assay systems are currently in use for the discovery of new modulators of cell growth, and in particular, in the search for new anti-cancer drugs which are specifically toxic to cancer cells but not to normal cells. A variety of changes may be scored for, but the most common ones are reversion of the transformed phenotype, significant changes in cell morphology, or cytotoxicity. The assays include: (1) in vitro cytotoxicity assays; (2) soft agar colony formation assays; (3) in vitro anti-microbial assays; and (4) assays which detect changes in cellular morphology.

In vitro cytotoxicity assays involve the measurement of cellular parameters which are indicative of inhibition of cellular growth or cytotoxicity. These include, for example, the measurement of the inhibition of certain cellular metabolic pathways in response to treatment with cytotoxic agents. The papers by Von Hoff, et al. (1985), and Catino, et al. (1985) describe typical methods which use this technique. These methods are somewhat complex technically, and require the use of radioactive tracers in some cases. Furthermore, the results are non-specific since any agent which alters the growth properties of cells will score positively in these assay systems.

Agents have also been tested for their ability to inhibit transformed (cancerous) cells from growing in soft agar. This method is based upon the finding by Freedman and Shin (1974) that the formation of colonies of cells in soft agar is the in vitro test which shows the highest correlation in predicting whether the cells will be tumorigenic in an experimental animal. This method is relatively simple to perform since colony growth will, after two or more weeks, generally be large enough to be seen with the naked eye. Scoring the final results, therefore, can be performed either by a technician without extensive training in tissue culture, or, as we describe in the current application, by an automated absorbance detection system. In its present form, however, this method is also non-specific for the same reasons as described above. In other words, any agent which inhibits cellular growth in any way will scores positively in this assay system as it is currently used, whether or not it inhibits the protein of interest.

In vitro anti-microbial assays involve the use of bacterial or yeast strains which are used as test organisms for screening for agents with generalized growth inhibitory properties (also described in Catino, et al., 1985). In this method, the bacterial or yeast strain is grown on standard media plates and potential agents are applied to various spots on the plates. If an agent has growth inhibitory properties, a clear zone results at the site of its application on the plate, resulting from the inability of the test strain to grow in the area. This method is rapid and can be performed by a technician without extensive training in tissue culture techniques, but the results are generally non-specific because agents which are effective against bacterial or yeast strains are frequently less effective (or completely ineffective) in modulating the growth of mammalian cells, as shown in the paper by Catino et al. (1985).

Still other screening systems depend upon a morphologic alteration of the test cells by exposure to the potential agents in order to determine the effectiveness of a given agent. This method is currently the most effective one for developing specific agents which interact with a given protein or alter a specific cellular property, as evidenced by the representative paper by Uehara, et. al. (1985). However, these screening systems are the most difficult ones to apply in practice, since the morphologic effect of each individual agent on the test cells must be studied under the microscope. Hence this method requires extensive observations of the cells by a trained scientist.

SUMMARY OF THE INVENTION

The Method presented in detail in this application combines the rapidity and ease of performance of the soft agar assay with a specificity for detecting an active agent exceeding that of the morphology assay. In brief, the method which we describe herein involves the generation of a cell line purposefully engineered to detect both stimulatory and inhibitory agents which are absolutely specific for any given protein which affects the cultural or morphological characteristics of the cell.

The basis for this invention is my observation that if a protein (the "protein of interest", or POI) which is involved in some manner in cellular growth control is overproduced in cells, then pharmacologic agents which can activate or inhibit the POI can result in altered growth properties of the cells.

The sensitivity of the cell is dependent on their production of the POI, a phenomenon referred to herein as a "graded cellular response" to the pharmacologically active agent.

The present invention provides a rapid, yet powerful screening system for the discovery and identification of both inhibitors and activators of proteins. The method may be applied to virtually any type of protein, including enzymes, receptors, DNA- or RNA-binding proteins, or others which are directly or indirectly involved in regulating cellular growth.

The method involves the insertion of a DNA (or cDNA) sequence encoding the Protein Of Interest (POI) into an appropriate vector and the generation of cell lines which contain either (1) the expression vector alone ("control" cell lines) or (2) the expression vector containing the inserted DNA (or cDNA) sequence encoding the POI ("test" cell lines). Using the appropriate vector system, recipient cell lines, and growth conditions, test cell lines can thus be generated which stably overproduce the corresponding POI. Under the appropriate growth conditions, these cell lines will exhibit a "graded cellular response" to activators or inhibitors of the POI. A screening system can thus be set up whereby the control and test cell lines are propagated in defined growth conditions in tissue culture dishes (or even in experimental animals) and large numbers of compounds (or crude substances which may contain active compounds) can be screened for their effects on the POI.

Substances which inhibit or activate the POI may affect characteristics such as growth rate, tumorigenic potential, anti-tumorigenic potential, anti-metastatic potential, cell morphology, antigen expression, and/or anchorage-independent growth capability. Substances which specifically inhibit or inactivate the POI may be distinguished from substances which affect cell morphology or growth by other mechanisms in that they will have a greater effect on the test lines than on the control lines.

The system has been tested using several cDNA sequences and several recipient cell lines, and can be easily automated.

The appended claims are hereby incorporated by reference as an enumeration of the preferred embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows the full-length cDNA sequence, and the deduced amino acid sequence, of one of several forms of PKC which has previously been isolated (cDNA clone RP58), and whose partial sequence has been reported (Housey, et al., 1987). It corresponds to PKCbetaI according to the nonenclature of Ono, et al. (1987). The deduced amino acid sequence begins with the first in-frame methionine codon at nucleotide position 91 and encodes a 671 amino acid protein with a predicted molecular weight of 76.8 kd. A consensus polyadenylation signal is underlined.

FIG. 1B shows the retrovirus-derived cDNA expression vector, developed in this laboratory, which was used for the present studies. The full-length RP58 cDNA encoding PKCbetaI (shown in 1A) was cloned into the Eco RI site of plasmid pMV7. The shaded region represents the coding sequence. "E" and "P" designate Eco RI and Pst I restriction sites, respectively. The indicated sizes between restriction sites in the RP58 cDNA are given in kilobases. "LTR" designates the 5' (left) and 3' (right) long terminal repeats of Moloney murine leukemia virus, and "TK-neo" designates the promoter region of the HSV thymidine kinase gene linked to the 5' end of the bacterial neomycin phosphotransferase (neo) gene.

FIG. 1-C outlines in schematic form the overall strategy used to generate cell lines stably overproducing PKC.

FIG. 2. Purification and Autophosphorylation of PKC. PKC activity from each cell line was purified and subjected to reaction conditions favoring autophosphorylation of PKC. Following the autophosphorylation reaction, protein samples were separated by discontinuous polyacrylamide gel electrophoresis. In the lanes bearing odd numbers the reaction mixtures contained 1 mM Ca2+ and phosphatidylserine to activate PKC, and in the lanes bearing even numbers the reaction mixtures contained 1 mM EGTA, 100 ng/ml TPA, and phosphatidylserine. The numbers in the left margin indicate the sizes of molecular weight markers, in kd. Arrows indicate the position of the 75 kd autophosphorylated PKC.

FIG. 3. Northern Blot Hybridization Analyses. Poly A+ RNA was isolated from the indicated cell lines and separated by electrophoresis on 6% formaldehyde/1% agarose gels, blotted onto nylon membrane and hybridized with the 32P-labelled full-length RP58 cDNA probe, as previously described (Housey, et. al., 1987). The numbers in the right margin indicate the sizes in kb of the RNA markers. The R6-PKC4 sample displayed a very weak 4.8 kb band on the original autoradiograph.

FIG. 4. Morphologic Responses of the Cell Lines to Phorbol Ester Treatment. Nearly confluent cultures of the three indicated cell lines were exposed to 100 ng/ml TPA in 0.1% dimethylsulfoxide (DMSO) solvent ("+TPA") or 0.1% DMSO alone ("−TPA"), in DMEM plus 10% CS. Photographs were taken 24 hours later (Panels A and D) and 48 hours later (Panels B and E). Fresh medium plus or minus TPA was then added and photographs were then taken an additional 24 hours later (Panels C and F). (Magnification : 100×).

FIG. 5. Growth Curves of Control and PKC-Overproducing Cell Lines. The indicated cell lines were seeded at 1×10^4 per 6 cm plate in DMEM plus 10% CS, in the presence ("+TPA") and absence ("−TPA") of 100 ng/ml TPA. Cell numbers were determined in replicate plates during the subsequent 11 day growth period. The values given indicate the means of triplicate determinations, which varied by less than 10%.

FIG. 6. Post-confluence Foci Formation. Control R6-C2 cells (Panel A) and R6-PKC3 cells (Panels B and C) were grown to confluence and then maintained for an additional 28 days in DMEM plus 10% CS (without TPA), with the addition of fresh medium every 3 days. Photographs were taken at the end of the 28 day period. Magnification : 40×.

FIG. 7. Growth in Soft Agar. Cell lines R6-C1, R6-PKC3, and R6-PKC5 were seeded into 60 mm petri dishes in 0.3% agar containing DMEM plus 20% FBS and 50 lg/ml G418, plus or minus 100 ng/ml TPA. Photographs were taken after 21 days of growth. (A) R6-C1+TPA (low-power field) (B) R6-C1+TPA (medium-power field) (C) R6-PKC3 (D) R6-PKC3 +TPA (E) R6-PKC5 (F) R6-PKC5+TPA. For additional details see Experimental Procedures. (Magnification : 100× in panel B; 40× in all other panels).

DETAILED DESCRIPTION OF THE INVENTION

The present method is intended for use in identifying potential chemical inhibitors or activators of enzymes, receptors, or any proteins which have effects upon cell phenotype. This method requires two cell lines, preferably alike except for their expression (production) of the protein of interest at different levels (and any further differences necessitated by that difference in expression). Inhibitors or activators are identified by their greater effect on the phenotype of the higher producing cell line.

Any phenotypic characteristic of the cell which is affected by expression of the protein of interest, other, of course, than the level of the protein itself, may be assayed. The phenotypic characteristic is preferably a "cultural" or "morphological" characteristic of the cell. For purposes of the appended claims, these terms are defined as follows:

Cultural characteristics include, but are not limited to the nutrients required for growth, the nutrients which, though not required for growth, markedly promote growth, the physical conditions (temperature, pH, gaseous environment, osmotic state, and anchorage dependence or independence) of the culture which affect growth, and the substances which inhibit growth or even kill the cells.

Morphological characteristics, but are not limited to include the size and shape of cells, their arrangements, cell differentiation, and subcellular structures.

Where the protein of interest is implicated in tumorigenesis or related phenomena,, the characteristic observed is preferably one related to cellular growth control, differentiation, de-differentiation, carcinogenic transformation, metastasis, tumorigenesis, or angiogenesis.

Phenotypic changes which are observable with the naked eye are of special interest. Changes in the ability of the cells to grow in an anchorage-independent manner, to grow in soft agar, to form foci in cell culture, and to take up selected stains are particularly appropriate phenomena for observation and comparison.

The higher producing cell line is preferably obtained by introducing a gene encoding the Protein of Interest (POI) into a host cell. The gene may be a one isolated from the genome of an organism, a cDNA prepared from an mRNA transcript isolated from an organism, or a synthetic duplicate of a naturally occurring gene. It may also have a sequence which does not occur exactly in nature, but rather corresponds to a mutation (single or multiple) of a naturally occurring sequence. No limitation is intended on the manner in which this mutated sequence is obtained. The gene is operably linked to a promoter of gene expression which is functional in the host, such that the corresponding Protein Of Interest (POI) is stably "overproduced" in the recipient cells to differing degrees. The promoter may be constitutive or inducible. By "overproduced", I mean that the POI is expressed at higher levels in the genetically manipulated cell line than in the original cell line. This allows one to generate cell lines which contain (or secrete) from as little as a few fold to more than 100-fold elevated levels of the POI relative to the control cells.

Any method may be used to introduce the gene into the host cell, including transfection with a retroviral vector, direct transfection (e.g., mediated by calcium phosphate or DEAE-dextran), and electroporation. Preferably, a retroviral vector is used

The host cells should exhibit a readily observable phenotypic change as a result of enhanced production of the POI. Preferably, this response should be proportional to the level of production of the POI. Finally, the cells should not spontaneously manifest the desired phenotypic change. For example, 3T3 cells form foci spontaneously. Among the preferred cell lines are Rat-6 fibroblasts, C3H10T ½ fibroblasts, and HL60. (*HL60* is a human cell line that differentiates in response to PKC activation.) 3T3 cells may be used, but with the reservation stated above.

Generally speaking, it is preferable to maximize the ratio of production by the "overproducing" cell line to production by the "native" line. This is facilitated by selecting a host cell line which produces little or no POI, and introducing multiple gene copies and/or using high signal strength promoters.

The Rat 6 embryo fibroblast cell line is a variant of the rat embryo fibroblast cell line established by Freeman et. al., (1972) and isolated by Hsiao et al., 1986. This cell line has an unusually flat morphology, even when maintained in culture at post-confluence for extended periods of time, displays anchorage dependent growth and, thus far, has not undergone spontaneous transformation. It was also ideal for these studies since it has a very low level of endogenous PKC activity and a low level of high affinity receptors for phorbol esters.

While my most preferred host cell line is the Rat-6 fibroblast cell line, I have tested this Method with other cell types, including the mouse NIH-3T3 cell line as well as the C3H 10T½ cell line. Tables 1(*a*) and (*b*) below show the representative specific activities of seven NIH-3T3 and six C3H-10T½ cell lines stably overproducing PKC. I have also recently performed the same experiments with the human HeLa cell line. In each case the resulting cell lines all exhibited growth properties qualitatively identical to those described for the PKC-expressing Rat-6 fibroblast cell lines. Therefore these results clearly demonstrate that many different types of cells can be employed in this method. The experimental procedures used to generate these cell lines were also identical to those used in connection with the Rat-6 cell line.

What we are looking for is a increase in the phenotypic change exhibited by the cell which becomes greater with increasing expression of the POI. We call this a "graded cellular response," and it is by this specialized response that we distinguish inhibitors or activators of the POI from agents that act upon other cell metabolites to effect a phenotypic change.

Thus, in a preferred embodiment, the cell lines are assayed for their relative levels of the POI, and their ability to grow in anchorage-independent systems (e.g., matrices such as soft agar or methocel), to form small "foci" (areas of dense groups of cells clustered together and growing on top of one another) in tissue culture dishes, to take up selected stains, or to bind an appropriately labeled antibody or other receptor for a cell surface epitope. In addition to exhibiting these growth control abnormalities, such cell lines will also be sensitive in their growth properties to chemical agents which are capable of binding to, or modifying the biological effects of, the POI.

The method is particularly unique in that it can be employed to search rapidly for EITHER activators OR inhibitors of a given POI, depending upon the need. The term "activators," as used herein, includes both substances necessary for the POI to become active in the first place, and substance which merely accentuate its activity. The term "inhibitors" includes both substance which reduce the activity of the POI and these which nullify it altogether. When a POI has more than one possible activity. The inhibitor or activator may modulate any or all of its activities.

The use of this screening method to identify inhibitors or activators of enzymes is of special interest. In particular, I am interested in using it to identify inhibitors or activators of enzymes involved in tumorigenesis and related phenomena, for example, protein kinase C, ornithine decarboxylase, cyclic AMP-dependent protein kinase, the protein kinase domains of the insulin and EGF receptors, and the enzyme products of various cellular onc genes such as the c-src ($PP60^{src}$) or c-ras ($P20^{ras}$) genes.

Protein kinase C (PKC) is a Ca^{2+}—and phospholipid-dependent serine/threonine protein kinase of fundamental importance in cellular growth control. PKC is activated endogenously by a wide variety of growth factors, hormones, and neurotransmitters, and has been shown to be a high affinity receptor for the phorbol ester tumor promoters as well as other agents which possess tumor promoting activity (for reviews see Nishizuka 1986; 1984; Ashendel, 1984). PKC has been shown to phosphorylate several intracellular protein substrates, including the epidermal growth factor (EGF) receptor (Hunter et al., 1984), pp60src (Gould et al., 1985), the insulin receptor (Bollag et al., 1986), p21 ras (Jeng et al., 1987), and many others (Nishizuka,

1986). Several laboratories have recently isolated cDNA clones encoding distinct forms of PKC, thus demonstrating that PKC is encoded by a multigene family (Ono et al., 1986, Knopf et al., 1986, Parker et al., 1986; Coussens et al., 1986; Makowske et al., 1986; Ohno et al., 1987; Housey et al., 1987). The multiple forms of PKC exhibit considerable tissue specificity (Knopf, et. al., 1986; Brandt et al., 1987; Ohno, et al, 1987; Housey, et. al., 1987) which suggests that there may be subtle differences in the function(s) of each of the distinct forms. However, all of the cDNA clones which have been isolated thus far that encode distinct forms of PKC share at least 65% overall deduced homology at the amino acid level, and transient expression experiments with some of these cDNA clones have shown that they encode serine/threonine protein kinase activities which bind to, or are activated by, the phorbol ester tumor promoters (Knopf, et. al., 1986, Ono, et. al., 1987).

We used the PKCbetaI cDNA clone for the present studies for the following reasons. With the exception of the brain, where its expression is very high, PKCbetaI is expressed at very low levels in most tissues, and its expression is virtually undetectable in Rat 6 fibroblasts (see below). Thus, we reasoned that using this form would maximize the phenotypic differences observed between control cells and cells overexpressing the introduced form of PKC. The PKCbetaI form is also of particular interest because within the PKC gene family its deduced carboxy terminal domain displays the highest overall homology to the catalytic subunit of the cyclic AMP-dependent protein kinase (PKAc) and the cyclic GMP-dependent protein kinase (PKG) (Housey et al., 1987). The latter observation suggests that PKAc, PKG, and the betaI form of PKC may share a common ancestral serine/threonine protein kinase progenitor, and that the additional PKC genes may have been derived through evolutionary divergence from the betaI form.

Agents which interact with certain structural proteins, such as actin and myosin, are also of interest. Mutations in the genes expressing these proteins may be involved in tumorigenesis and metastasization. Such interactions can lead to changes in cell phenotype which can be assayed by this method.

As is set forth in greater detail below, I have produced cell lines which overproduce protein kinase C (PKC). These cell lines, unlike the control cells, grow in soft agar even in the absence of the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA has been shown to be a potent activator of PKC. When TPA is added to the growth medium the PKC-overproducing cell lines grow even better and form considerably larger colonies in soft agar. Furthermore, I have also tested known inhibitors of PKC activity which, as predicted, caused the PKC-overproducing cells to grow less well (or not at all) in soft agar. Thus, the direct utility of this method in identifying both activators or inhibitors of a gene product, in this case PKC, has been clearly demonstrated.

In additional studies with other genes, most notably the c-H-ras oncogene, the catalytic subunit of the cyclic AMP-dependent protein kinase, the c-myc oncogene, and certain cDNA clones encoding phorbol-ester inducible proteins, similar results have been obtained. Thus it is also clear that the method can be generalized to a wide variety of genes encoding proteins which are involved in cellular growth control in numerous cell types.

First I tested the capability of pMV7-based expression vectors (pMV7 is my preferred transfer vector) to produce several different types of proteins in various cell lines. I used the cDNA sequences encoding the following proteins: hypoxanthine/guanine phosphoribosyltransferase (HGPRT), the human T4 lymphocyte cell surface antigen, the human T8 lymphocyte cell surface antigen, and ornithine decarboxylase (ODC). In each case the pMV7 vector was capable of producing high levels of expression of the relevant gene thereby resulting in overproduction of the corresponding protein product.

Once I had verified that the pMV7-derived expression vector could reproducibly generate cell lines which stably overproduced proteins I then tested additional genes which encode proteins, other than PKC, which are also involved in cellular growth control.

In further experiments, I have tested the method using an activated c-H-ras oncogene (T24), again in analogous fashion to the techniques described herein, and again with analogous results to those described herein for the PKC cDNA clone Thus, the Method can also be used for the rapid development of a p21 ras inhibitor. Taken together, the results described in this application demonstrate directly that the Method described herein is clearly generalizable to any gene which is involved in any way in cellular growth control.

The preferred protein inhibitor/activator drug screening method of the present invention comprises the following steps:

1. Construction of an expression vector which is capable of expressing the protein of interest in the selected host by inserting a gene encoding that protein into a transfer vector. The gene may be inserted 3' of a promoter already borne by the transfer vector, or a gegne and a promoter may be inserted sequentially or simultaneously.

2. Introduction of the expression vector (a) into cells which produce recombinant retrovirus particles, or (b) directly into host cells which will be used for subsequent drug screening tests (the resulting cells are called herein "test" cells).

In parallel, the transfer vector (i.e., the vector lacking the gene of interest and possibly a linked promoter but otherwise identical to the expression vector) is preferably also introduced into the host cells. Cell lines derived from this latter case will be used as negative controls in the subsequent drug screening tests. Alternatively, the unmodified host cells may be used as controls.

If *2a* was employed, after an appropriate time (usually 48 hours), media containing recombinant virus particles is transferred onto host cells so as to obtain test or control cells.

3. The test and control cells are transferred to selective growth medium containing the appropriate drug which will only allow those cells harboring the expression vector containing the selectable marker gene (as well as the gene or cDNA of experimental interest) to grow. After an appropriate selection time (usually 7–10 days), individual clones of cells (derivative cell lines) are isolated and propagated separately.

4. Each independent cell line is tested for the level of production of the POI. By this method, a range of cell lines is generated which overprod..ce from a few fold to more that 100-fold levels of the POI. In parallel, the

control cell lines which contain only the transfer vector alone (with the selectable marker gene) are also assayed for their endogenous levels of the POI.

5. Each independent line is then tested for its growth capability in soft agar (or methocel, or any other similar matrix) of various percentages and containing different types of growth media until cell lines are identified which possess the desired growth characteristics as compared to the control cell lines.

6. Each cell line is also tested for its ability to form "foci", or areas of dense cellular growth, in tissue culture plates using media containing various percentages and types of serum (20%, 10%, 5% serum, fetal calf serum, calf serum, horse serum, etc.) and under various conditions of growth (e.g. addition of other hormones, growth factors, or other growth supplements to the medium, temperature and humidity variations, etc.). In these tests, the cells are maintained at post-confluence for extended periods of time (from two to eight weeks) with media changes every three days or as required. Such growth parameters are varied until cell lines are identified which possess the desired foci formation capacity relative to the control cell lines under the identical conditions.

7. After a cell line possessing the required growth characteristics is identified, the cells are grown under the conditions determined in (5) above with the growth medium supplemented with either crude or purified substances which may contain biologically active agents specific to the POI. Thus, crude or purified substances possessing the latter properties can be rapidly identified by their ability to differentially alter the growth properties of the experimental cells (which overproduce the POI) relative to the control cells (which do not). This can be done rapidly even by simple observation with the naked eye, since the colonies which grow in soft agar after 2 weeks are easily seen even without staining, although they may be stained for easier detection.

Similarly, if the post-confluence foci formation assay is chosen, the foci which result after approximately two weeks can be easily seen with the naked eye, or these foci can also be stained. For screening very large numbers of compounds (tens of thousands or more), the entire procedure can be performed on 96 well tissue culture plates. This applies equally well for either the soft agar growth assay or the tissue culture foci formation assay. Results of the assays can be rapidly determined by measuring the relative absorbance of the test cells as compared to the control cells (at 500 nm, or another appropriate wavelength). Absorbance readings may be rapidly performed in a 96-well plate absorbance reader such as the "Titer-tek" plate reader, or any of several analogous apparatus currently available. In this fashion, thousands of compounds could be screened per month for their biological activity with very low labor and materials costs.

Furthermore, if antigen expression varies on the test cells expressing high levels of the POI relative to the control cells, a simple Enzyme-Linked Immunosorbption Assay (ELISA) could be performed and an antibody specific to the antigen.

While the assay may be performed with one control cell line and one test cell line, it is possible to use additional lines, tests lines with differing POI levels. Also additional sets of control/test lines, originating from other hosts, may be tested.

Specific examples implementing the series of steps described above are as follows.

EXAMPLE 1

If one were interested in screening for a protein kinase C (PKC) inhibitor, cell lines would be generated and selected which grow well in soft agar (as a result of their overproduction of any form of PKC) and yet show an enhancement of their growth when compounds which are known to stimulate PKC are added to the growth medium. Appropriate control cells, of course, would not exhibit any of these characteristics. Screening for a potent PKC inhibitor could then be performed by searching for those substances which could selectively inhibit the soft agar (anchorage-independent) growth of the PKC-overproducing cell lines. Alternatively, since the PKC-overproducing cells also form small, dense foci in tissue culture, one could also screen for substances which inhibit this foci formation.

Described below are the detailed aspects of the relevant techniques and methods used to apply the principles of the invention to the problem of developing a system useful for screening for potent inhibitors of protein kinase C (PKC), a high-affinity intracellular receptor for tumor-promoting agents. The cell lines which resulted from the application of this method are highly sensitive and responsive both to agents which activate PKC as well as to those which inhibit PKC.

Construction of plasmid pMV7

The construction of pMV7 was begun with plasmid pPyori which contains the polyoma virus origin of replication cloned into the unique BamHI site of pML-1 (Lusky and Botchan, 1981). This plasmid replicates in murine cells that contain the polyoma T antigen (Dailey and Basilico, 1985). Plasmid pMV (Perkins et al., 1983) was then cleaved with HincII and BglI. The 2.29 kb fragment that contains the Moloney Leukemia Virus Long Terminal Repeats (LTR), the packaging site, the splice donor site, and the proline tRNA binding site was isolated.

During the original construction of pMV (Perkins et al., 1983) 3.95 kb was removed from the MSV genome by cleaving with PstI. This left a PstI site situated 380 bps 3' from the tRNA binding site and 308 bps from the 5' end of the 3' LTR (Reddy et. al., 1981). Xho I linkers were added to the HincII-BglI fragment, and to pPyori after it had been cleaved with EcoRI and HindIII. The two fragments were ligated, after activation of the linkers, and a plasmid, designated pMV-3, that contained the Moloney Virus control elements was isolated.

The unique EcoRI site was removed from this vector by digesting the plasmid with EcoRI and treating the linear molecules with T4 polymerase. These molecules were recircularized and a plasmid, pMV-4, lacking the EcoRI site, was isolated. An EcoRI linker was inserted into this plasmid at the PstI site between the Moloney LTRs; the resultant plasmid was designated pMV-5.

The dominant selectable marker (neo) was added to pMV-5. The first step was isolating a 1.9 kb BamHI-SalI fragment from pIPB1. This fragment contains the Herpes Simplex virus thymidine kinase (tk) promoter region and the coding sequence for the bacterial neo gene (neomycin phosphotransferase). This fragment was blunt-ended with T4 polymerase, ClaI linkers were added and the fragment was cloned into the Cla I site 165 bps 3' to the EcoRI site in pMV-5, between the

LTRs. This plasmid was designated pMV5-tk neo. The tk promoter has an EcoRI site 79 bps 5' to the start of transcription. This EcoRI site was removed by partially digesting pMV5-tkneo with EcoRI, isolating the linear full length cut species, filling in the ends with T4 polymerase and recircularizing the molecule. A plasmid was chosen in which the EcoRI site previously present in the tk promoter sequence was removed, but the EcoRI site 537 bps 3' to the start of transcription of the 5' LTR was retained. This plasmid was designated pMV7 and a map of this plasmid is shown in FIG. 1B.

This vector consists of the 5' and 3' Moloney Murine Leukemia Virus (MoMuLV) LTRs, the MoMuLV RNA packaging site 3' to the 5' LTR, an Eco RI cloning site, a modified Herpes Simplex Virus thymidine kinase (tk) promoter (lacking an Eco RI site at −79 bp), and the selectable marker gene neo. cDNA clones inserted into the EcoRI cloning site are under the transcriptional control of the 5' LTR, whereas the neo gene is independently transcribed by the tk promoter. This structure favors maintenance of the functional integrity of the selectable marker without interfering with the expression of the 5' (unselected) cDNA sequence. It is known (Maddon et al., 1986; Daley et al., 1987) that when various cDNA sequences are inserted into the EcoRI site of pMV7 they can be readily transferred into recipient cells by virus-mediated passage, are stably expressed, and yield high-level production of the corresponding protein.

Nucleotide Sequencing and Expression Vector Construction

Nucleotide sequencing of the PKC cDNA clone RP58, a full-length clone isolated from a rat brain cDNA library, which corresponds to the previously reported clone RP41, was performed as previously described (Housey et al., 1987). The full-length cDNA sequence of RP58, which encodes PKCbeta1 (FIG. 1A), was subcloned into the EcoRI site of plasmid pMV7 using standard methodology (Maniatis et al., 1983). The general structure of pMV7 is shown in FIG. 1B. The construct resulting from insertion of the PKCbeta1 gene is designated pMV7-PKCbeta1.

Isolation of cell lines stably overexpressing PKC

20 ug of CsCl banded pMV7 or pMV7-PKCbeta1 plasmid DNA were transfected (Graham and van der Eb, 1973; as modified by Wigler et al., 1977) onto subconfluent "Psi-2" cells (Mann et al., 1985). After 48 hrs the culture medium was collected, filtered through a 0.45 um filter and stored at −70° C. Recipient subconfluent Rat-6 fibroblasts (5×10^5 per 10 cm plate) were infected with the virus-containing medium in 2 ug/ml polybrene for 48 hrs. The cells, grown to confluence, were then trypsinized and replated in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (CS) (Flow Laboratories) with 200 ug/ml of the neomycin derivative G418 (Geneticin). Resistant colonies were cloned by ring isolation after 1 week of G418 selection.

After approximately ten days of growth in selective medium, ten individual G418-resistant clones were isolated and maintained independently in G418-containing medium. These lines were designated R6-PKCbeta11 through R6-PKCbeta110 (abbreviated as R6-PKC1 through R6-PKC10). In parallel, a set of control Rat 6 lines was generated by transfection of the plasmid pMV7 (lacking the PKC cDNA insert) onto W-2 cells, infection of recipient Rat-6 cells, and selection for G418 resistance as described above for plasmid pMV7-PKCbeta1. Similarly, after ten days of growth in the G418-containing medium, five individual, well-isolated G418-resistant clones were then isolated and maintained independently. These control lines were designated R6-C1 through R6-C5.

RNA Isolation and Blot Hybridization Analyses

Poly A+ RNA isolation, gel electrophoresis, and blot hybridization analyses were performed as previously described (Housey, et. al., 1985). RNA molecular weight markers were obtained from Bethesda Research Laboratories. The 2.7 kb cDNA insert of RP58 (see above) was subcloned into plasmid pKS(+) (Stratagene Cloning Systems) to yield a plasmid designated pS2-RP58. A ^{32}P-labelled probe was prepared from pS2-RP58 and used under high-stringency hybridization conditions as previously described (Housey et al., 1987).

Purification and Assay of PKC Activity From Tissue Culture Cells

The total PKC activity (membrane-associated plus cytosolic) present in cultured cells was determined after partial purification of cellular extracts as follows.

Three 10 cm plates of confluent cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS) and then 10 ml of homogenization buffer (20 mM Tris, pH 7.5, 5 mM EGTA, 5 mM EDTA, 15 mM 2-mercaptoethanol, 10 ug/ml soybean trypsin inhibitor, 10 ug/ml leupeptin, 40 ug/ml phenylmethylsulfonyl fluoride), containing 0.1% Triton X-100 were added. The cells were then scraped from each of the plates, pooled and disrupted with 25 strokes in a Dounce homogenizer. The homogenate was transferred to a 15 ml disposable polystyrene tube, centrifuged at 2000×g for 5 minutes at 4° C., and the supernatant was loaded onto a 1 ml DEAE Sephacel column previously equilibrated with 10 ml homogenization buffer, at 4° C. The column was washed with 10 ml homogenization buffer and then the bound enzyme was eluted with 3 ml of homogenization buffer containing 0.5 M NaCl. Total protein concentrations were determined by the method of Bradford (1976).

The PKC activity present in the above-described partially purified cell extracts above was assayed immediately after isolation. The synthetic peptide R-K-R-T-L-R-R-L, corresponding to amino acids 651–658 of the epidermal growth factor receptor (Ullrich et al., 1984), was synthesized on an Applied Biosystems model 430A peptide synthesizer, purified by high-performance liquid chromatography, lyophilized, and stored at −20° C. The Threonine at position 654 is an in vivo substrate for PKC (Hunter, et al., 1984; Davis and Czech, 1985). This synthetic peptide is a highly specific substrate for PKC activity in vitro. (Watson et al., 1987; Woodgett, et al., 1986).

The purified material was then redissolved in sterile water at a final concentration of 100 uM and used as the phosphoacceptor substrate in the PKC assays. The general method of assay has been published in detail elsewhere (O'Brian et al., 1985). In most cases, 100 uM synthetic peptide was substituted for 2 mg/ml histone III-S as the phosphoacceptor substrate.

Cell extracts were prepared 48 hours after the cells had reached confluence. As shown in Table 1(*a*), eight of the ten cell lines generated by infection with the pMV7-PKCbeta1 construct (lines R6-PKC1, R6-PKC3,

and R6-PKC5 through R6-PKC10) contained marked increases in PKC activity when compared to the control lines (R6-C1, -C2, and -C3). It is remarkable that cell line R6-PKC3 contained a 53 fold higher level of PKC activity than that present in the control cells. Two of the lines (R6-PKC2 and R6-PKC4), however, did not display a significant increase in PKC activity, yet they presumably had integrated the pMV7-PKC construct as evidenced by their continued G418 resistance. Subsequent studies verified that these two cell lines contained deletions in the cDNA clone encoding PKCbetaI.

In additional studies I found that the very high PKC activity in extracts of R6-PKC3 seen in the presence of 1 mM Ca^{2+} and phosphatidylserine was also apparent in the presence of 1 mM EGTA, 100 ng/ml TPA and phosphatidylserine. This very high activity was also seen when we employed histone III-S, rather than the above-described synthetic peptide, as the substrate for phosphorylation. Furthermore, even after 24 weeks of continuous growth and serial passage, the cell lines R6-PKC1 through R6-PKC6 displayed essentially the same levels of PKC activity shown in Table 1.

Autophosphorylation and Gel Electrophoresis of PKC

Cell extracts purified as described above were incubated under conditions which favor the autophosphorylation of PKC, as follows. One hundred ug of partially purified protein extract was incubated in a reaction mixture containing 80 ug/ml phosphatidylserine, 1 mM CaCl2 (or 1 mM EGTA and 100 ng/ml TPA), 5 mM MgCl2, and 30 uM ATP containing 100 uCi [gamma-^{32}P]ATP (New England Nuclear, NEG035). The purity of the radioactive ATP is critical to obtain reproducible autophosphorylation of PKC.

Under these conditions, it has been previously shown that PKC undergoes an autophosphorylation reaction which results in the phosphorylation of several sites on the intact enzyme (Walton et al., 1987; Huang et al., 1986, Woodgett and Hunter, 1986; Kikkawa, 1982).

Reactions were incubated at room temperature for 10 minutes and then stopped by the addition of SDS-PAGE loading buffer containing 2-mercaptoethanol. This material was then subjected to discontinuous SDS-PAGE by a modification of the method of Laemmli (1970). Twenty ug of total protein were loaded onto each lane. Following electrophoresis, the gels were fixed in 50% acetic acid, 10% ethanol, dried, and autoradiographed on Kodak XAR-5 film.

Autoradiographs of these gels (FIG. 2) revealed that the extracts prepared from four cell lines that had high PKC activity (R6-PKC1, -PKC3, -PKC5, and -PKC6, see Table 1(*a*)) displayed a prominent phosphorylated protein band which was about 75 kd in size, corresponding to the size of an autophosphorylated preparation of PKC obtained from rat brain (Huang et al., 1986; Housey et al., 1987). When examined in an immunoblot assay this 75 kd band also reacted with an antibody to the betaI form of PKC (Jaken and Kiley, 1987). The control cell lines R6-C1, -C2 and -C3, and the cell lines R6-PKC2 and R6-PKC4, which did not have increased levels of PKC (see Table 1(*a*)) did not show this 75 kd phosphorylated band (FIG. 2), nor did they contain any bands which reacted with the antibody to the betaI form of PKC. It is of interest that the samples obtained from the four cell lines that produced high levels of PKC also displayed weaker but distinct phosphorylated protein bands that were about 73, 60, and 49 kd in size, which were not seen (or only faintly detected) in the extracts from cells that did not have increased levels of PKC (FIG. 2). These bands may represent degradation fragments of the 75 kd PKC molecules, or specific cellular proteins that are phosphorylated by PKC.

The above-described phosphorylated protein bands were seen when either 1 mM Ca2+ plus phosphatidylserine or 100 ng/ml TPA plus phosphatidylserine were used as cofactors for PKC activation (compare even and odd numbered lanes in FIG. 2). When, however, extracts from cell lines producing high levels of PKC were incubated in an autophosphorylation reaction in the absence of such cofactors, the 75 kd band and the additional smaller bands described above were not detected. These results, taken together with the negative results obtained with extracts from the control cells (FIG. 2), clearly indicate that the phosphorylated bands reflect PKC activity.

Phorbol Ester (^{3}H-PDBU) Binding Assays

Since it has been shown that PKC is a high affinity intracellular receptor for the phorbol ester tumor promoters (for review, see Nishizuka, 1986), I also assayed a subset of the cell lines for 3H-PDBu binding using a previously described intact cell assay (Horowitz et al., 1981).

Cells were plated at 1×10^5 per 4 cm well on day 1, the medium was changed on day 2 and the cells then assayed on day 3. The monolayer was washed with 4 ml of DMEM (without serum), then 2 ml of DMEM containing 50 nM ^{3}H-PDBU (New England Nuclear; 8.3 Ci/mmol) was added and the plates were incubated for ½ hr at 37° C. to determine total binding. The fraction of the total binding that represented specific binding was determined by the addition of a 1000-fold excess of unlabeled PDBU (LC Services) to the 3H-PDBU stock (Horowitz et al., 1981). The plates were washed 3× with 4 ml ice-cold PBS. The cells were solubilize in 1 ml 1% SDS/10 mM DTT for 2 hrs at 37° C. The lysate was transferred to a scintillation vial and counted. Replica plates were used to determine the number of cells per plate and the specific binding data expressed as nanomoles 3H-PDBu bound/10^6 cells. Scatchard analyses were performed as previously described (Horowitz et al., 1981).

I found that the R6-PKC cell lines 1,3,5 and 6, all of which had high PKC enzyme activity, also had a marked increase in 3H-PDBu binding, whereas the cell line R6-PKC4, which did not display a significant increase in PKC activity, did not show an increase in 3H-PDBu binding when compared to the two control cell lines R6-C1 and R6-C2 (Table 1(*a*)). Scatchard analyses of the control cell line R6-C1 and of the R6-PKC3 cell line, performed as previously described (Horowitz et al., 1981), indicated that the number of high affinity receptors in the two cell lines was 1.6×10^5 and 1.4×10^6, respectively. The affinity constants were approximately the same in both cell lines (Kd=16 nM). Thus, under the assay conditions used, the R6-PKC3 cells contain about ten times the level of high affinity phorbol-ester binding sites as the control cells. It is apparent that the cell lines that express very high levels of PKC also have a significant increase in phorbol ester binding sites.

Assays for PKC-related RNA transcripts

In view of the above results, it was of interest to analyze the poly A+ RNA fraction of several of the cell lines described in Table 1(*a*) for the size and abun-

dance of RNA transcripts containing sequences homologous to PKCbetaI.

Poly A+ RNA was separated on 1% agarose, 6%-formaldehyde gels, blotted onto nylon membranes, hybridized to a 32P-labelled DNA probe prepared from the full-length PKCbetaI cDNA, and autoradiographed, as previously described (Housey, et al., 1986). As shown in FIG. 3, the lines that contained elevated levels of PKC activity (R6-PKC1,-PKC3,-PKC5, and -PKC6, see Table 1(*a*)) contained a prominent 6.6 kb RNA species which corresponds to the predicted size for a mRNA transcript that initiates in the 5' LTR and terminates in the 3' LTR of the pMV7-PKCbetaI construct. This transcript was most abundant in the R6-PKC3 cell line (FIG. 3) which also expresses the highest level of PKC activity (Table 1).

On the other hand, lines R6-PKC2 and R6-PKC4, which showed no significant elevation of PKC activity (Table 1(*a*)), produced truncated mRNA's of approximately 5 kb and 4.8 kb, respectively. The abundance of the latter transcripts was much lower than that of the 6.6 kb transcripts present in the cell lines that expressed high levels of PKC. The neo+ phenotype and the lack of PKC activity in cell lines R6-PKC2 and R6-PKC4 suggest that the truncated mRNAs reflect deletions in PKC coding sequences in the integrated constructs carried by these cell lines. Indeed, genomic DNA blot hybridizations of clones R6-PKC2 and R6-PKC4 indicated that they contained deletions in the PKCbetaI cDNA sequence. Neither in the parental Rat 6 cell line, nor in any of the experimental cell lines derived from these cells, was it possible to detect evidence of an endogenous transcript homologous to the PKCbetaI probe (FIG. 3). Thus, in these cells, there is negligible expression of the endogenous gene encoding PKCbetaI.

Assays of Growth in Monolayer Culture and in Soft Agar

Cells were seeded at a density of 10^4/plate in a series of 60 mm plates, in 5 ml DMEM plus 10% CS. Twenty-four hours later, cells in triplicate plates were trypsinized and counted. This point was designated "day 0." The remaining cultures were then grown in the respective medium (i.e. plus or minus 100 ng/ml TPA) with fresh medium changes twice per week. Cell counts per plate were then determined on triplicate plates during the remainder of the growth curve (FIG. 3). The results obtained were analyzed for exponential doubling time and saturation density (Table 2). To assess growth in soft agar (anchorage independence), 2×10^4 cells were suspended in 2 ml of 0.3% Bacto agar (Difco Laboratories, Detroit, Mich.) in DMEM containing 20% fetal calf serum (FCS) and overlayed above a layer of 5 ml of 0.5% agar in the same medium, on 60 mm petri dishes. The cells were then overlaid with DMEM plus 20% FCS every 4 days. At the end of 30 days, colonies were stained with the vital stain 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolinium chloride hydrate (INT) (Sigma Chemical Co., St. Louis, Mo.) for 48 hours at 37° C., in an incubator with 5% CO2 (Schaeffer and Friend, 1976), and the number of colonies counted under low power on an inverted phase microscope. The data are expressed as "cloning efficiency", i.e. number of colonies greater than 0.05 mm per plate ×100 divided by the number of cells originally seeded per plate.

Screening of a Known Activator of PKC

We found that cell lines which overproduce PKC display an exaggerated morphologic response to 12-0-tetradecanoyl phorbol-13-acetate (TPA) and altered growth control.

Changes in Morphology

To further characterize the phenotypic changes which occurred in the cell lines that overproduce PKCbetaI, lines R6-PKC3 and R6-PKC5, which contain 53- and 20-fold elevations of PKC activity, respectively, were first examined in detail with respect to their morphology, in comparison to the control cell line R6-C1. As shown in FIG. 4, Panel A, in the absence of TPA treatment all three cell lines showed the characteristic fusiform morphology of monolayer cultures of the normal parental Rat 6 fibroblast cell line. At 24 hours after treatment with 100 ng/ml TPA (FIG. 4, R6-C1: panel D), the control cell line displayed more elongated and dendritic cells and a criss-cross pattern, changes previously seen shortly after rodent fibroblasts are treated with TPA (Boreiko et al., 1980). These changes were, however, much more dramatic when the R6-PKC3 and R6-PKC5 cells were treated with TPA (R6-PKC3 and R6-PKC5, Panel D). This was particularly striking with the R6-PKC3 cells (which express the highest level of PKC) since they displayed very long cytoplasmic processes and numerous refractile cell bodies.

By 48 hours following exposure to TPA the morphology of the control cell line R6-C1had returned to its normal appearance (FIG. 4, Panel E). On the other hand, the R6-PKC3 cells, and to a lesser extent the R6-PKC5 cells, continued to display an altered morphology. All of the cell lines were then exposed to a second, fresh dose of TPA (100 ng/ml) and examined 24 hours later (FIG. 4, Panel F), i.e., 72 hours after the first dose of TPA. The control cells failed to respond, in terms of morphologic change, to the second dose of TPA whereas the R6-PKC3 cells continued to display their altered morphology as well as an increase in cell density. The R6-PKC5 cells displayed only slight changes in morphology in response to the second dose of TPA.

Presumably, the very high level of constitutive production of PKC in the R6-PKC3 cell line is responsible for their exaggerated morphologic response to TPA as well as the failure of these cells to display the usual refractory response to TPA following an initial exposure. In normal cells, the latter response appears to be due to "down-regulation" of endogenous PKC activity. Since the R6-PKC5 cells have an intermediate level of PKC, it is not surprising that their morphologic responses to TPA are intermediate between those of the control cells and the R6-PKC3 cells.

Growth Curves

It was also of interest to determine the growth rates of these cells in monolayer culture. Detailed growth curves were performed on R6-C1, R6-PKC3 and R6-PKC5 cells in 10% calf serum and DMEM medium, in the absence and presence of 100 ng/ml TPA. The data obtained are summarized quantitatively in Table 2.

In the absence of TPA, the R6-C1 control cell line displayed the longest doubling time (26.4 hours) and the lowest saturation density (3.4×10^6 cells/plate); the R6-5 cell line had a shorter doubling time (24.9 hours) and a higher saturation density (4.8×10^6 cells/plate); and the R6-PKC3 cell line had the shortest doubling

time (24.2 hours) and the highest saturation density (5.7×10^6 cells/plate). The presence of TPA decreased the doubling and also increased the saturation densities of all three cell lines, but the enhancement by TPA was particularly striking in the case of R6-PKC3 (Table 2).

When the cell lines were maintained in the presence of TPA for a longer period of time, the R6-PKC3 cells, but not the R6PKC5 or R6-C1 cells, showed a decline in cell density. The latter effect was due to the fact that when the R6-PKC3 cells were maintained at high cell density in the presence of TPA they became less adhesive and tended to detach from the plate.

Thus, even in the absence of TPA the R6-PKC3 cells, (which have the highest level of PKC), and to a lesser extent the R6-PKC5 cells (which have an intermediate level of PKC), exhibit an enhancement of their growth properties which is even greater than that seen when the R6-C1 control cells are grown in the presence of TPA. Moreover, in the presence of TPA these differences in growth properties between the control and R6-PKC3 cells are even more striking.

Foci Formation

In additional studies, monolayer cultures were maintained at post-confluence for an extended period of time (28 days), with media changes every 3 days, in the absence of TPA. Whereas the control R6-C2 cell line remained a fairly uniform monolayer, after about 21 days the R6-PKC3 cell line developed numerous dense foci which were approximately 0.1–0.3 mm in diameter (FIG. 5). Furthermore, the R6-PKC3 cultures displayed numerous cells with a highly vacuolated cytoplasm which were scattered throughout the monolayer, but were not seen in the R6-C2 control culture. When the dense foci seen in the R6-PKC3 culture were picked and further passaged they grew like the parental R6-PKC3 cells and did not display a morphology typical of malignantly transformed cells. It may be surmised that these dense foci, and the vacuolated cells, reflect physiologic rather than genetic changes induced by the high level of PKC activity.

Growth on Soft Agar

I also assayed these cell lines for their ability to form colonies in soft agar, since with rodent cells the acquisition of anchorage-independent growth often correlates with tumorigenicity (Freedman and Shin, 1974). As shown in FIG. 6, when 2×10^4 cells were plated in 0.3% soft agar, both the R6-PKC3 and the R6-PKC5 cells formed numerous small colonies, whereas the control R6-C1 cells (and the parental Rat 6 cell line) failed to grow and persisted as single cells. In addition, when TPA (100 ng/ml) was added to the agar medium, the colony sizes and cloning efficiencies of the R6-PKC3 and RC-PKC5 cells were enhanced (FIG. 6 and Table 2), but the R6-C1 cells still failed to grow in agar.

The cloning efficiencies and colony sizes of the R6-PKC3 cultures were always greater than those of the R6-PKC5 cultures, both in the absence and presence of TPA (Table 2), presumably reflecting the higher level of PKC activity in the former cell line. Thus, it is clear that the overproduction of PKC is associated with the acquisition of anchorage-independent growth in Rat 6 cells. The sizes of the colonies formed in agar by the PKC cell lines are smaller than those formed by Rat 6 cells transformed by an activated c-H-ras oncogene, which have a diameter of about 0.5–1.0 mm.

Further Screening of Activators and Inhibitors of PKC

Additional compounds tested included the tumor promoters teleocidin, aplysiatoxin, and mezerein, all of which are known activators of PKC (O'Brian, et al., CSH, 1985). Furthermore, a known inhibitor of PKC, H-7 (Kawamoto and Hidaka, 1984), also modulated the growth of the cells in the expected manner.

Moreover, this method has been used to establish that the anti-estrogen tamoxifen (O'Brian, et al, Cancer Res., 1985), which inhibits PKC enzyme activity in a cell-free assay, is capable of completely inhibiting the growth in agar of all of the cell lines overproducing PKC. Inhibition of the growth of the R6-PKC3 cells in agar in the presence of tamoxifen provided critical evidence that tamoxifen could inhibit PKC-mediated stimulation of cellular growth.

Furthermore, the concentration of the inhibitor necessary to completely inhibit the growth of each cell line was roughly proportional to the amount of PKC being overproduced in that particular cell line. In other words, there is a direct relationship between the molar amount of inhibitor required to prevent cell growth and the molar amount of PKC present in each cell line.

In addition, I have demonstrated the analogous relationship between the molar amounts of PKC activators and the molar amounts of PKC present in cells. In other words, there is a direct relationship between the molar amount of activator required to stimulate cell growth and the molar amount of PKC present in cells. Thus, this work establishes, for the first time, the fact that stable overproduction of a protein in mammalian cells can result in a novel cellular phenotype(s) (in this case anchorage independence) which can be directly modulated by chemical agents which interact with the protein.

EXAMPLE 2

If one were interested in screening for a potent inhibitor of the c-H-ras oncogene product (the p21 protein) then one would generate cells which grow well in soft agar with appropriate media conditions when p21 is stably overproduced at a certain level, but not at all when p21 is present at wild type levels. Screening for a potent p21inhibitor could then be performed as described in Example 1 above.

EXAMPLE 3

The same basic techniques would also apply to genes (or cDNA sequences) which have been mutated either by laboratory design (e.g. site-directed mutagenesis) or as a result of naturally occurring events. Thus, any of the known point mutations in the ras oncogene which result in greater capability by the mutated gene to transform normal cells to cancerous ones could be employed in the same basic procedures as described above.

Further Modifications

Use of any expression vector capable of stably overexpressing a given gene in a recipient cell could be used with success in the procedures described herein. The retroviral vector which I used here was particularly well suited to the problem since I had designed it specifically for these purposes. However, other similar vector systems would work. Also, one could do co-transfection of an experimental gene inserted in one plasmid vector along with a second plasmid containing the selectable marker gene (rather than having both the experimental gene and the selectable marker gene on

the same plasmid vector). This is more difficult and less efficient than using the pMV7 vector, but it would work to some extent.

Any growth medium, in addition to soft agar or methocel, which tends to prohibit the growth of normal, non-transformed cells, could also be used.

A culture of *E. coli* DH1 bearing the plasmid denoted pMV7-RP58 (pMV7-PKC beta1), was deposited under the Budap Treaty with the American Type Culture Collection on Feb. 11, 1988, and redeposited Mar. 17, 1988, then receiving ATCC No. 67654. The deposit of this plasmid is not to be construed as an admission that the deposit is required for enablement or that the disclosure is limited to the deposited vector or gene.

TABLE 1(a)

PKC Activity and Phorbol Ester Binding in Rat 6 Cells Infected with pMV7 or pMV7-PKCbeta1 Constructs

	PKC Activity		
Cell Line	Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control	3H-PDBU Binding (pmol/10^6 cells)
R6-C1	100	—	1.6
R6-C2	85	—	1.3
R6-C3	150	—	1.5
mean + s.d.	100 + 34	1	
R6-PKC1	2480	23	12.7
R6-PKC2	85	1	ND
R6-PKC3	5840	53	9.9
R6-PKC4	190	2	0.7
R6-PKC5	2200	20	5.8
R6-PKC6	4600	42	7.1
R6-PKC7	2150	20	ND
R6-PKC8	3280	30	ND
R6-PKC9	4990	45	ND
R6-PKC10	5050	46	ND

TABLE 1(b)

PKC Activity in NIH-3T3 Cell Lines Infected with pMV7 or pMV7-PKCbeta1 Constructs

	PKC Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control
NIH-3T3 Cell lines (Controls)		
3T3-C1	110	—
3T3-C2	150	—
3T3-C3	90	—
mean +/− s.d. (control lines)	115 +/− 30	
NIH-3T3 PKC-Overproducing Cell Lines		
3T3-PKC1	2570	22
3T3-PKC2	3640	32
3T3-PKC3	1960	17
3T3-PKC4	1340	11
3T3-PKC5	4190	36
3T3-PKC6	2110	18
3T3-PKC7	5050	44

TABLE 1(c)

PKC Activity in C3H-10T1/2 Cell Lines Infected with pMV7 or pMV7-PKCbeta1 Constructs

	PKC Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control
C3H-10T1/2 Cell lines (Controls)		
C3H-C1	115	—
C3H-C2	155	—
C3H-C3	130	—
C3H-C4	185	—
mean +/− s.d.	145 +/− 30	
C3H-10T1/2 PKC-Overproducing Cell Lines		
C3H-PKC1	2340	16
C3H-PKC2	5010	35
C3H-PKC3	950	7
C3H-PKC4	1360	9
C3H-PKC5	4340	30
C3H-PKC6	7460	51

Legend to Table 1

Control cell lines were obtained by infecting rat-6-fibroblast, NIH-3T3 and C3H-10T1/2 cells with the pMV7 vector itself (lacking the PKC cDNA insert) whereas the PKC-overproducing cell lines were obtained from rat-6-fibroblast, NIH-3T3 and C3H-10T1/2 cells infected with the pMV7-PKCbeta1 construct, as described above. Total PKC activity was partially purified from each of the cell lines and assayed in the presence of 1 mM $Ca2+$ and 80 ug/ml phosphatidylserine, using the synthetic peptide R—K—R—T—L—R—R—L as substrate. Specific activity is reported as the amount of incorporation of 32P into the synthetic peptide substrate per milligram of protein per minute. All assays were done in duplicate and varied by less than 10%.

TABLE 2

GROWTH PROPERTIES OF RAT 6 CELL LINES OVER-PRODUCING PKC AND THEIR RESPONSES TO TPA TREATMENT

		Growth in Culture			
		Monolayer		Agar	
Cell Line	TPA Add	Doubling Time (hrs)	Saturat. Density ($\times 10^6$)	Effic. (%)	Colony Size (mm)
R6C1	—	26.4	3.4	0	—
(control)	+	24.6	4.2	0	—
R6-PKC3	—	24.2	5.7	25.1	0.10-0.15
(test)	+	21.5	10.0	29.7	0.15-0.35
R6-PKC5	—	24.9	4.8	17.3	0.05-0.10
(test)	+	22.9	7.0	34.7	0.10-0.15

Table 2. The cells were grown as described above. The "doubling time" relates to the initial exponential phase of cell growth and the "saturation density" represents the number of cells per 6 cm plate on day 11. The data are taken from the experiment shown in FIG. 3.

TABLE 3

Inhibition of Growth in Agar of PKC-Overproducing Cell Lines Using Various Inhibitors

Inhibitor	Cell Line	Conc. Inhibitor (uM)	Effic. of Growth in Agar (%)
H-7	R6-PKC3	0	27
H-7	R6-PKC3	2	23
H-7	R6-PKC3	5	22
H-7	R6-PKC3	10	17
H-7	R6-PKC3	50	4
H-7	R6-PKC3	100	0
H-7	R6-PKC5	0	20
H-7	R6-PKC5	2	21
H-7	R6-PKC5	5	16
H-7	R6-PKC5	10	4
H-7	R6-PKC5	50	1
H-7	R6-PKC5	100	0
Tamoxifen	R6-PKC3	0	25
Tamoxifen	R6-PKC3	5	27
Tamoxifen	R6-PKC3	20	22
Tamoxifen	R6-PKC3	50	15
Tamoxifen	R6-PKC3	100	6
Tamoxifen	R6-PKC3	200	0
Tamoxifen	R6-PKC5	0	19
Tamoxifen	R6-PKC5	5	19
Tamoxifen	R6-PKC5	20	17
Tamoxifen	R6-PKC5	50	8

TABLE 3-continued

Inhibition of Growth in Agar of PKC-Overproducing Cell Lines Using Various Inhibitors

Inhibitor	Cell Line	Conc. Inhibitor (μM)	Effic. of Growth In Agar (%)
Tamoxifen	R6-PKCS	100	0
Tamoxifen	R6-PKCS	200	0

References

Ashendel, C. The Phorbol Ester Receptor: a phospholipid-regulated protein kinase. (1984) Biochim. Biophys. Acta 822, 219–242.

Bollag, G. E., Roth, R. A., Beaudoin, J., Mochly-Rosen, D., Koshland, D. E. Jr. (1986) Protein kinase C directly phosphorylates the insulin receptor in vitro and reduces its protein-tyrosine kinase activity. Proc. Natl. Acad Sci. USA 83, pp 5822–4.

Boreiko, C., Mondal, S., Narayan, S., and Heidelberger, C. (1980) Effect of 12-O-Tetradecanoylphorbol-13-acetate on the Morphology and Growth of C3H/10T½ Mouse Embryo Cells. Cancer Res. 40, 4709–4716.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brandt, S. J., Niedel, J. E., Bell, R. M., Young, W. S. (1987) Distinct patterns of expression of different protein kinase C mRNAs in rat tissues. Cell 49, pp 57–63.

Catino, J. J., Francher, D. M., Edinger, K. J., and Stringfellow, D. A. (1985). A microtitre cytotoxicity assay useful for the discovery of fermentation-derived antitumor agents. Cancer Chemother. Pharmacol. 15, 240–243.

Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U., & Ullrich, A. (1986) Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. Science 233, 859–866.

Dailey, L., and Basilico, C. (1985). Sequences in the polyomavirus DNA regulatory region involved in viral DNA replication and early gene expression. J. Virol. 54, 739–749.

Daley, G. Q., McLaughlin, J., Witte, O. N., Baltimore, D. The CML-Specific P210 bcr/abl Protein, Unlike v-abl, Does Not Transform NIH/3T3 FibroblastsScience 237, pp 532–535.

Davis, R. J., and Czech, M. P. (1985) Platelet-derived growth factor mimics phorbol diester action on epidermal growth factor receptor phosphorylation at threonine 654. Proc. Natl. Acad. Sci. 82, 4080–4084.

Freeman, A. E., Price, P. J., Igel, H. J., Young, J. C., Maryak, J. M. Huebner, R. J. (1970) Morphological transformation of rat embryo cells induced by diethylnitrosamine and murine leukemia viruses. J. Natl. Cancer Inst. 44, pp 65–78.

Freedman, V. H. and Shin, S. (1974) Cellular Tumorigenicity in nude Mice: Correlation with Cell Growth in Semi-Solid Medium. Cell 3, 355–359.

Graham, F. L., and van der Eb, A. J. (1973) A new technique for the assay of infectivity of human adenovirus DNA. Virology 52, 456–467.

Gould, K. L., Woodgett, J. R., Cooper, J. A., Buss, J. E., Shalloway, D., Hunter, T. (1985) Protein Kinase C Phosphorylates pp60src at a novel site. Cell 42, pp 849–857.

Horowitz, A. D., Greenebaum, E. and Weinstein, I. B. (1981) Identification of receptors for phorbol ester tumor promoters in intact mammalian cells and of an inhibitor of receptor binding in biologic fluids. Proc. Natl. Acad. Sci. USA 78, pp 2315–2319.

Housey. G. M., O'Brian, C. A., Johnson, M. D., Kirschmeier, P., and Weinstein, I. B. (1987) Isolation of cDNA clones encoding protein kinase C: Evidence for a protein kinase C-related gene family. Proc. Natl. Acad. Sci. USA 84, pp 1065–1069.

Housey, G. M., Kirschmeier, P., Garte, S. J., Burns, F., Troll, W., & Weinstein, I. B. (1985) Expression of long terminal repeat (LTR) sequences in carcinogen-induced murine skin carcinomas. Biochem. Biophys. Res. Commun. 127, 391–398.

Hsiao, W.-L. W., T. Wu, Weinstein, I. B., (1986) Oncogene-Induced Transformation of a Rat Embryo Fibroblast Cell Line is Enhanced by Tumor Promoters. Mol. Cell. Biol. 6, pp 1943–1950.

Hunter, T., Ling, N., Cooper, J. A. (1984) Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature 311, 480–3.

Huang, K. P., Nakabayashi, H., Huang, F. L. (1986) Isozymic forms of rat brain Ca2+-activated, phospholipid-dependent protein kinase. Proc. Natl. Acad. Sci. 83, 8535–8539.

Jaken, S. and Kiley, S. (1987) Purification and characterization of three types of protein kinase C from rabbit brain cytosol. Proc. Natl. Acad. Sci. USA 84, pp 4418–4422.

Jeng, A. Y., Srivastava, S. K., Lacal, J. C., Blumberg, P. M. (1987) Phosphorylation of ras oncogene product by protein kinase C. Biochem. Biophys. Res. Commun. 145, pp 782–8.

Johnson, M. D., Housey, G. M., Kirschmeier, P., and Weinstein, I. B. (1987) Molecular Cloning of Gene Sequences Regulated by Tumor Promoters Through Protein Kinase C. Mol. Cell Biol. 7, 2821–2829.

Kajikawa, N., Kishimoto, A., Shiota, M., & Nishizuka, Y. (1983) Ca2+-dependent neutral protease and proteolytic activation of Ca2+-activated, phospholipid-dependent protein kinase. Methods. Enzymol. 102, 279–290.

Kawamoto, S. and Hidaka, H. (1984) 1-(5-Isoquinolinesulfonyl) -2-methyl-piperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. Biochem. Biophys. Res. Commun. 125, 258.

Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982) Calcium-activated, phospholipid-dependent protein kinase from rat brain. Subcellular distribution, purification, and properties. J. Biol. Chem. 257, 13341–13348.

Kirschmeier, P. T., Housey, G. M., Johnson, M. D., Perkins, A. S., and Weinstein, I. B. (1988) Construction and Characterization of a Retroviral Vector Demonstrating Efficient Expression of Cloned cDNA Sequences. DNA, in press.

Knopf, J. L., Lee, M-H, Sultzman, L. A., Kriz, R. W., Loomis, C. R., Hewick, R. M. & Bell, R. (1986) Cloning and expression of multiple protein kinase C cDNAs. Cell 46, 491–502.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Leach, K. L., James, M. L., & Blumberg, P. M., (1983) Characterization of a specific phorbol ester

aporeceptor in mouse brain cytosol. Proc. Natl. Acad. Sci. USA 80, 4208–4212.

Lusky, M., and Botchan, M. (1981). Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. Nature 293, 74–81.

Maddon, P. J., Dalgleish, A. G., McDougal, J. S., Clapham, P. R., Weiss, R. A., Axel, R. (1986) The T4 Gene Encodes the AIDS Virus Receptor and Is Expressed in the Immune System and the Brain. Cell 47, pp 333–348.

Makowske, M., Birnbaum, M. J., Ballester, R., Rosen, O. M. (1986) A cDNA encoding PKC identifies two species of mRNA in brain and GH3 cells. J. Biol. Chem. 261, pp 13389–13392.

Maniatis, T., Fritsch, E. F., & Sambrook, J. eds. (1983) in Molecular Cloning : A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.)

Mann, R., Mulligan, R. C., and Baltimore, D. (1983) Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell 33, 153–159.

Nishizuka, Y. (1986) Studies and Perspectives of Protein Kinase C. Science 233, 305–312. Nishizuka, Y. (1984) The Role of Protein Kinase C in Cell Surface Transduction and Tumour Promotion. Nature (London) 308, 693–698.

O'Brian, C. A., Lawrence, D. S., Kaiser, E. T., & Weinstein, I. B. (1984) Protein kinase C phosphorylates the synthetic peptide Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val in the presence of phospholipid plus either Ca2+or a phorbol ester tumor promoter. Biochem. Biophys. Res. Commun. 124, 296–302.

O'Brian, C., Arcoleo, J., Housey, G. M., & Weinstein, I. B. (1985) in Cancer Cells 3, eds. Feramisco, J., Ozanne, B. & Stiles, C. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) 359–363.

O'Brian, C. A., Liskamp, R. M., Solomon, D. H. and Weinstein, I. B. (1985) Inhibition of Protein Kinase C by Tamoxifen. Cancer Res 45, 2462–2465.

Ono, Y., Kurokawa, T., Fujii, T., Kawahara, K., Igarashi, K., Kikkawa, U., Ogita, K., Nishizuka, Y. (1986) Two types of complementary DNAs of rat brain protein kinase C. FEBS 206, 347–52.

Ono, Y., Kikkawa, U., Ogita, K., Tomoko, F., Kurokawa, T., Asaoka, Y, Sekiguchi, K., Ase, K., Igarashi, K., Nishizuka, Y. (1987) Expression and Properties of Two Types of Protein Kinase C: Alternative Splicing from a Single Gene. Science 236, pp 1116–1120.

Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokohura, H., Sakoh, T., Hidaka, H. (1987) Tissue-specific expression of three distinct types of rabbit protein kinase C. Nature (London) 325, pp 161–6.

Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., & Ullrich, A. (1986) The complete primary structure of protein kinase C–the major phorbol ester receptor. Science 233, 853–858.

Perkins, A. S., Kirschmeier, P. T., Gattoni-Celli, S., and Weinstein, I. B. (1983). Design of a retrovirus-derived vector for expression and transduction of exogenous genes in mammalian cells. Mol. Cell. Biol. 3, 1123–1132.

Pontremoli, S., Melloni, E., Michetti, M., Sparatore, B., Salamino, F., Sacco, O., and Horecker, B. L. (1987) Phosphorylation and proteolytic modification of specific cytoskeletal proteins in human neutrophils stimulated by phorbol-12-myristate 13-acetate. Proc. Natl. Acad. Sci. 84, 3604–3608.

Sibley, D. R., Benovic, J. L., Caron, M. G., Lefkowitz, R. J. (1987) Regulation of transmembrane signaling by receptor phosphorylation Cell 48, 913–922.

Uehara, Y., Hori, M., Takeuchi, T., Umezawa, H. (1985) Screening of Agents Which Convert 'Transformed Morphology' of Rous Sarcoma Virus-Infected Rat Kidney Cells to 'Normal Morphology': Identification of an Active Agent as Herbimycin and its Inhibition of Intracellular src Kinase. Jpn. J. Cancer. Res. 76, 672–675.

Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., and Seeburg, P. H. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309, 418–425.

Walton, G. M., Bertics, P. J., Hudson, L. G., Vedvick, T. S., Gill, G. N. (1987) A Three-Step Purification Procedure for Protein Kinase C: Characterization of the Purified Enzyme. Anal. Biochem. 161, 425–437.

Weinstein, I. B., (1987) Growth Factors, Oncogenes, and Multistage Carcinogenesis. J. Cell. Biochem. 33, pp 213–224.

Wigler, M., Silverstien, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C, and Axel, R. (1977). Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11, 223–232.

Woodgett, J. R., Gould, K. L., and Hunter, T. (1986) Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. Eur. J. Biochem. 161 177–184.

Young, S., Parker, P. J., Ullrich, A., and Stabel, S. (1987) Down-regulation of protein kinase C is due to an increased rate of degradation. Biochem. J. 244, 775–779.

Von Hoff, D. D., Forseth, B., and Warfel, L. E. (1985) Use of a Radiometric System to Screen for Antineoplastic Agents: Correlation with a Human Tumor Cloning System. Cancer Res. 45, 4032–4036.

I claim:

1. A method of determining whether a substance is an inhibitor or activator of a protein whose production by a cell evokes a responsive change in a phenotypic characteristic other than the level of said protein in said cell per se, which comprises:

(a) providing a first cell line which produces said protein and exhibits said phenotypic response to the protein;

(b) providing a second cell line which produces the protein at a lower level than the first cell line, or does not produces the protein at all, and which exhibits said phenotypic response to the protein to a lesser degree or not at all;

(c) incubating the substance with the first and second cell lines; and

(d) comparing the phenotypic response of the first cell line to the substance with the phenotypic response of the second cell line to the substance.

2. The method of claim 1 wherein the response is one observable with the naked eye.

3. The method of claim 1 wherein the response is a change in a cultural or morphological characteristic of the cell.

4. The method of claim 1 wherein the response is a change in the ability of the cell line to grow in an anchorage-independent fashion.

5. The method of claim 1 wherein the response is a change in the ability of the cell line to grow on soft agar.

6. The method of claim 1 wherein the response is a change in foci formation in cell culture.

7. The method of claim 1 wherein the response is a change the ability of the cells to take up a selected stain.

8. The method of claim 1 in which the protein is an enzyme.

9. The method of claim 8 wherein increased activity of the enzyme is correlated with increased tumorigenesis.

10. The method of claim 9 in which the enzyme is a protein kinase C enzyme or a fragment, domain, or subunit of a receptor which has protein kinase C activity.

11. The method of claim 9 wherein the enzyme is ornithine decarboxylase.

12. The method of claim 9 in which the protein is the expression product of an oncogene.

13. The method of claim 1 in which the substance is a suspected inhibitor of the biological activity of the protein.

14. The method of claim 1 in which the substance is a suspected activator of the biological activity of the protein.

15. The method of claim 1, wherein said first cell line is obtained by introducing a gene encoding the protein of interest into a host cell, said gene being under the control of a promoter functional in the host cell, whereby said gene is expressed.

16. The method of claim 15, wherein the gene is introduced into the host cell by means of a first genetic vector into which the gene has been inserted, and said second cell line is obtained by introducing into a similar host cell a second genetic vector essentially identical to the first genetic vector except that it does not bear said gene insert.

17. The method of claim 15 wherein the gene is introduced into the host cell by means of a retroviral vector.

18. The method of claim 15 in which the host cell line essentially does not produce the protein.

19. The method of claim 15 in which the host cell line is a rat-6 fibroblast cell line.

20. The method of claim 3 in which the response is a change in the differentistion state of the cell.

21. The method of claim 1 wherein the response is a change in an antigenic characteristic of the cell.

22. A test kit for determining whether a substance is an inhibitor or activator of a protein whose production evokes a responsive change in a phenotypic characteristic other than level of said protein in said cell per se, which comprises:

(a) a first animal cell line which produces the protein and exhibits said phenotypic response thereto; and

(b) a second animal cell line which produces the protein at a lower level than the first animal cell line, or does not produce the protein, and which exhibits said phenotypic response to the protein to a lesser degree or not at all.

23. The test kit of claim 22, wherein the level of production of the protein in the first cell line is at least five times the level of production of the protein in the second line.

24. The test kit of claim 22, wherein the phenotypic response to expression of the protein is selected from the group consisting of changes in growth rate, saturation density, plating efficiency in soft agar, colony size in soft agar, and combinations thereof.

* * * * *

US005266464A

United States Patent [19]

Housey

[11] Patent Number: 5,266,464

[45] Date of Patent: * Nov. 30, 1993

[54] METHOD OF SCREENING FOR PROTEIN INHIBITORS AND ACTIVATORS

[75] Inventor: Gerard Housey, New York, N.Y.

[73] Assignee: ICT Pharmaceuticals, Inc., New York, N.Y.

[*] Notice: The portion of the term of this patent subsequent to Dec. 25, 2007 has been disclaimed.

[21] Appl. No.: 392,073

[22] Filed: Aug. 10, 1989

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 154,206, Feb. 10, 1988, Pat. No. 4,980,281.

[51] Int. Cl.[5] C12Q 1/02

[52] U.S. Cl. 435/29; 435/240.1; 435/240.2

[58] Field of Search 435/29

[56] References Cited

U.S. PATENT DOCUMENTS

4,480,038 10/1984 Cheng 435/29

4,532,204 7/1985 Crespi et al. 435/29

Primary Examiner—Sam Rosen

Attorney, Agent, or Firm—Kenyon & Kenyon

[57] ABSTRACT

Inhibitors and activators of a protein whose expression affects a phenotypic characteristic of the cell, especially a cultural or morphological characteristic, are identified by their more pronounced effect on cells producing higher, usually non-naturally occurring, levels of the protein, than on cells producing little or none of the protein. In a preferred assay, the effect is observable with the naked eye.

By this method, tamoxifen is identified as an inhibitor of PKC activity in cell culture.

8 Claims, 22 Drawing Sheets

FIG. 1.

```
          10          20          30          40          50          60
          *           *           *           *           *           *
GAA TTC CGC CTC TCC GGG CTT ACA GCC CGC GGT CCC GCC GCC CCG GGG CCG CCA CCT CTC

          70          80          90          100         110         120
          *           *           *           *           *           *
GGG GCT CCC CCC AGT CCC CGC GCG CGC AAG ATG GCT GAC CCG GCT GCG GGG CCG CCG CCG
                                    Met Ala Asp Pro Ala Ala Gly Pro Pro Pro

          130         140         150         160         170         180
          *           *           *           *           *           *
AGC GAG GGC GAG GAG AGC ACG GTG CGC TTC GCC CGC AAA GGG CCC CTC CGG CAG AAG AAC
Ser Glu Gly Glu Glu Ser Thr Val Arg Phe Ala Arg Lys Gly Pro Leu Arg Gln Lys Asn
```

FIG. 1 CONT.

```
      190         200         210         220         230         240
       *           *           *           *           *           *
GTG CAC GAG GTG AAG AAC CAC AAA TTC ACC GCC CGC TTC TTC AAG CAG CCC ACC TTC TGC
Val His Glu Val Lys Asn His Lys Phe Thr Ala Arg Phe Phe Lys Gln Pro Thr Phe Cys

      250         260         270         280         290         300
       *           *           *           *           *           *
AGC CAC TGC ACC GAC TTC ATT TGG GGC TTC GGG AAG CAG GGA TTC CAG TGT CAA GTC TGC
Ser His Cys Thr Asp Phe Ile Trp Gly Phe Gly Lys Gln Gly Phe Gln Cys Gln Val Cys

      310         320         330         340         350         360
       *           *           *           *           *           *
TGC TTT GTT GTA CAC AAG CGC TGC CAT GAA TTC GTC ACG TTC TCC TGC CCT GGT GCA GAC
Cys Phe Val Val His Lys Arg Cys His Glu Phe Val Thr Phe Ser Cys Pro Gly Ala Asp
```

FIG. 1 CONT.

```
          370         380         390         400         410         420
           *           *           *           *           *           *
AAG GGC CCG GCC TCT GAT GAC CCA CGG AGC AAA CAC AAG TTT AAG ATC CAC ACC TAC TCC
Lys Gly Pro Ala Ser Asp Asp Pro Arg Ser Lys His Lys Phe Lys Ile His Thr Tyr Ser

          430         440         450         460         470         480
           *           *           *           *           *           *
AGC CCT ACC TTC TGT GAC CAC TGT GGA TCA CTG CTG TAT GGG CTC ATC CAC CAG GGG ATG
Ser Pro Thr Phe Cys Asp His Cys Gly Ser Leu Leu Tyr Gly Leu Ile His Gln Gly Met

          490         500         510         520         530         540
           *           *           *           *           *           *
AAA TGC GAC ACC TGT ATG ATG AAT GTC CAC AAG CGC TGC GTG ATG AAC GTC CCC AGC CTC
Lys Cys Asp Thr Cys Met Met Asn Val His Lys Arg Cys Val Met Asn Val Pro Ser Leu
```

FIG. 1 CONT.

```
          550         560         570         580         590         600
           *           *           *           *           *           *
TGT GGC ACC GAC CAC ACA GAA CGC CGT GGC CGC ATC TAC ATC CAG GCC CAC ATC GAC AGG
Cys Gly Thr Asp His Thr Glu Arg Arg Gly Arg Ile Tyr Ile Gln Ala His Ile Asp Arg

          610         620         630         640         650         660
           *           *           *           *           *           *
GAG GTC CTC ATC GTT GTT GTA AGA GAT GCT AAA AAT CTG GTA CCT ATG GAC CCC AAC GGC
Glu Val Leu Ile Val Val Val Arg Asp Ala Lys Asn Leu Val Pro Met Asp Pro Asn Gly

          670         680         690         700         710         720
           *           *           *           *           *           *
TTG TCA GAT CCC TAC GTA AAA CTG AAA CTG ATC CCT GAT CCC AAA AGT GAG AGC AAG CAG
Leu Ser Asp Pro Tyr Val Lys Leu Lys Leu Ile Pro Asp Pro Lys Ser Glu Ser Lys Gln
```

FIG. 1 CONT.

```
          730         740         750         760         770         780
           *           *           *           *           *           *
AAG ACC AAG ACT ATC AAA TGC TCC CTC AAC CCG GAG TGG AAC GAA ACC TTC AGA TTT CAG
Lys Thr Lys Thr Ile Lys Cys Ser Leu Asn Pro Glu Trp Asn Glu Thr Phe Arg Phe Gln

          790         800         810         820         830         840
           *           *           *           *           *           *
CTG AAG GAA TCA GAC AAA GAC AGA AGA CTG TCC GTA GAG ATC TGG GAT TGG GAC CTG ACC
Leu Lys Glu Ser Asp Lys Asp Arg Arg Leu Ser Val Glu Ile Trp Asp Trp Asp Leu Thr

          850         860         870         880         890         900
           *           *           *           *           *           *
AGC AGG AAT GAC TTC ATG GGA TCT CTG TCG TTT GGG ATT TCA GAA CTA CAG AAA GCC GGA
Ser Arg Asn Asp Phe Met Gly Ser Leu Ser Phe Gly Ile Ser Glu Leu Gln Lys Ala Gly
```

FIG. 1 CONT.

```
          910         920         930         940         950         960
           *           *           *           *           *           *
GTG GAT GGC TGG TTC AAG TTA CTA AGC CAG GAA GAA GGC GAG TAC TTT AAT GTG CCG GTG
Val Asp Gly Trp Phe Lys Leu Leu Ser Gln Glu Glu Gly Glu Tyr Phe Asn Val Pro Val

          970         980         990        1000        1010        1020
           *           *           *           *           *           *
CCG CCG GAA GAA AGC GAG GGC AAT GAA GAG CTG CGG CAG AAG TTT GAG AGA GCC AAG ATT
Pro Pro Glu Glu Ser Glu Gly Asn Glu Glu Leu Arg Gln Lys Phe Glu Arg Ala Lys Ile

         1030        1040        1050        1060        1070        1080
           *           *           *           *           *           *
GGC CAA GGT ACC AAG GCT CCA GAA GAA AAG ACA GCG AAC ACT ATA TCC AAA TTT GAC AAC
Gly Gln Gly Thr Lys Ala Pro Glu Glu Lys Thr Ala Asn Thr Ile Ser Lys Phe Asp Asn
```

FIG. 1 CONT.

```
        1090        1100        1110        1120        1130        1140
          *           *           *           *           *           *
AAT GGC AAC AGG GAC CGG ATG AAA CTG ACC GAT TTT AAC TTC CTG ATG GTG CTG GGG AAA
Asn Gly Asn Arg Asp Arg Met Lys Leu Thr Asp Phe Asn Phe Leu Met Val Leu Gly Lys

        1150        1160        1170        1180        1190        1200
          *           *           *           *           *           *
GGC AGC TTT GGC AAG GTC ATG CTC TCA GAG CGG AAG GGT ACA GAT GAA CTC TAT GCC GTG
Gly Ser Phe Gly Lys Val Met Leu Ser Glu Arg Lys Gly Thr Asp Glu Leu Tyr Ala Val

        1210        1220        1230        1240        1250        1260
          *           *           *           *           *           *
AAG ATC CTG AAG AAA GAT GTG GTG ATC CAA GAT GAC GAT GTG GAG TGC ACA ATG GTG GAG
Lys Ile Leu Lys Lys Asp Val Val Ile Gln Asp Asp Asp Val Glu Cys Thr Met Val Glu
```

FIG. 1 CONT.

```
        1270        1280        1290        1300        1310        1320
          *           *           *           *           *           *
AAG AGG GTG CTG GCC CTG CCT GGG AAG CCC CCA TTC CTG ACT CAG CTC CAT TCC TGC TTC
Lys Arg Val Leu Ala Leu Pro Gly Lys Pro Pro Phe Leu Thr Gln Leu His Ser Cys Phe

        1330        1340        1350        1360        1370        1380
          *           *           *           *           *           *
CAG ACC ATG GAC CGC CTC TAC TTT GTG ATG GAG TAT GTG AAC GGG GGC GAC CTC ATG TAC
Gln Thr Met Asp Arg Leu Tyr Phe Val Met Glu Tyr Val Asn Gly Gly Asp Leu Met Tyr

        1390        1400        1410        1420        1430        1440
          *           *           *           *           *           *
CAC ATC CAA CAA GTT GGC CGT TTC AAG GAG CCC CAT GCT GTA TTT TAC GCT GCA GAG ATT
His Ile Gln Gln Val Gly Arg Phe Lys Glu Pro His Ala Val Phe Tyr Ala Ala Glu Ile
```

FIG. 1 CONT.

```
        1450        1460        1470        1480        1490        1500
          *           *           *           *           *           *
GCC ATC GGT CTT TTC TTC TTG CAG AGC AAG GGC ATC ATT TAC CGT GAC CTG AAA CTT GAC
Ala Ile Gly Leu Phe Phe Leu Gln Ser Lys Gly Ile Ile Tyr Arg Asp Leu Lys Leu Asp

        1510        1520        1530        1540        1550        1560
          *           *           *           *           *           *
AAC GTG ATG CTG GAT TCC GAG GGG CAC ATC AAA ATC GCT GAC TTT GGC ATG TGT AAA GAG
Asn Val Met Leu Asp Ser Glu Gly His Ile Lys Ile Ala Asp Phe Gly Met Cys Lys Glu

        1570        1580        1590        1600        1610        1620
          *           *           *           *           *           *
AAT ATC TGG GAT GGG GTG ACA ACC AAG ACA TTC TGT GGC ACT CCA GAC TAC ATT GCC CCA
Asn Ile Trp Asp Gly Val Thr Thr Lys Thr Phe Cys Gly Thr Pro Asp Tyr Ile Ala Pro
```

FIG. 1 CONT.

```
        1630        1640        1650        1660        1670        1680
          *           *           *           *           *           *
GAG ATC ATT GCT TAT CAG CCC TAC GGA AAG TCT GTG GAC TGG TGG GCG TTT GGA GTC CTG
Glu Ile Ile Ala Tyr Gln Pro Tyr Gly Lys Ser Val Asp Trp Trp Ala Phe Gly Val Leu

        1690        1700        1710        1720        1730        1740
          *           *           *           *           *           *
CTG TAT GAA ATG TTG GCT GGC CAG GCA CCT TTT GAA GGG GAG GAT GAG GAT GAA CTC TTC
Leu Tyr Glu Met Leu Ala Gly Gln Ala Pro Phe Glu Gly Glu Asp Glu Asp Glu Leu Phe

        1750        1760        1770        1780        1790        1800
          *           *           *           *           *           *
CAG TCA ATC ATG GAG CAC AAC GTG GCG TAT CCC AAG TCC ATG TCT AAG GAA GCT GTG GCA
Gln Ser Ile Met Glu His Asn Val Ala Tyr Pro Lys Ser Met Ser Lys Glu Ala Val Ala
```

FIG. 1 CONT.

```
        1810          1820          1830          1840          1850          1860
          *             *             *             *             *             *
ATC TGC AAA GGG CTA ATG ACC AAA CAC CCA GGC AAG CGC CTG GGT TGT GGG CCT GAA GGG
Ile Cys Lys Gly Leu Met Thr Lys His Pro Gly Lys Arg Leu Gly Cys Gly Pro Glu Gly

        1870          1880          1890          1900          1910          1920
          *             *             *             *             *             *
GAA CGA GAC ATT AAG GAG CAT GCA TTT TTC CGG TAT ATC GAC TGG GAG AAA CTC GAA CGC
Glu Arg Asp Ile Lys Glu His Ala Phe Phe Arg Tyr Ile Asp Trp Glu Lys Leu Glu Arg

        1930          1940          1950          1960          1970          1980
          *             *             *             *             *             *
AAG GAG ATT CAG CCA CCT TAT AAA CCA AAA GCT AGA GAC AAG CGA GAC ACC TCC AAC TTC
Lys Glu Ile Gln Pro Pro Tyr Lys Pro Lys Ala Arg Asp Lys Arg Asp Thr Ser Asn Phe
```

FIG. 1 CONT.

```
        1990          2000          2010          2020          2030          2040
          *             *             *             *             *             *
GAC AAA GAG TTC ACC AGG CAG CCT GTG GAA CTG ACT CCC ACT GAC AAA CTC TTC ATC ATG
Asp Lys Glu Phe Thr Arg Gln Pro Val Glu Leu Thr Pro Thr Asp Lys Leu Phe Ile Met

        2050          2060          2070          2080          2090          2100
          *             *             *             *             *             *
AAC TTG GAC CAA AAT GAA TTT GCT GGC TTC TCG TAT ACT AAC CCA GAG TTT GTC ATT AAT
Asn Leu Asp Gln Asn Glu Phe Ala Gly Phe Ser Tyr Thr Asn Pro Glu Phe Val Ile Asn

        2110          2120          2130          2140          2150          2160
          *             *             *             *             *             *
GTG TAG GTG AAT GCA GAT TCC ATC GCT GAG CCT GTG TGT AAG GCT GCA GCG TGA ATG TCT
Val ---
```

FIG. 1 CONT.

```
      2170        2180        2190        2200        2210        2220
       *           *           *           *           *           *
ATT ATC AAT TCC AGT CTT CCA GGA TTC ATG GTG CCT CTG TTG GCA TCC GTC ATG TGG AGA

      2230        2240        2250        2260        2270        2280
       *           *           *           *           *           *
GCT TGT CTT AGA GGG CTT TTC TTT GTA TGT ATA GCT TGC TAG TTT GTT TTC TAC ATT TCA

      2290        2300        2310        2320        2330        2340
       *           *           *           *           *           *
AAA TGT TTA GTT TAG AAT AAG TGC ATT GCC CAC TGA TAG AGG TAC AAT TTT CCA GAC TTC
```

FIG. 1 CONT.

```
          2530          2540          2550          2560          2570          2580
            *             *             *             *             *             *
GTA AGC TTT GCA GTT ACT GTG AAC TCT TGT CTC TTG GAG GAA CTT TTT GTT TAA GAA TTG

          2590          2600
            *             *
GTA TGA TTA AAC TGA ATT C*
```

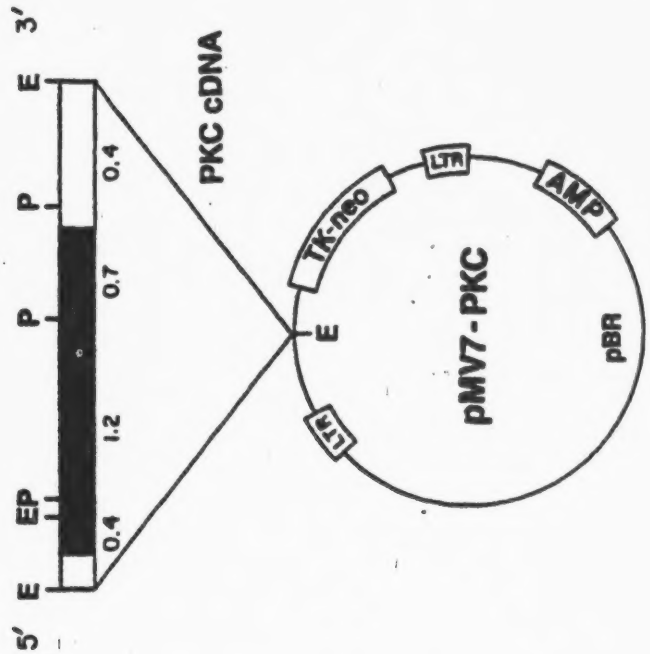

5'
E
EP
P
P
E
3'
0.4
1.2
0.7
0.4
PKC cDNA
E
pMV7-PKC
TK-neo
LTR
LTR
AMP
pBR

FIG. 1.
B.

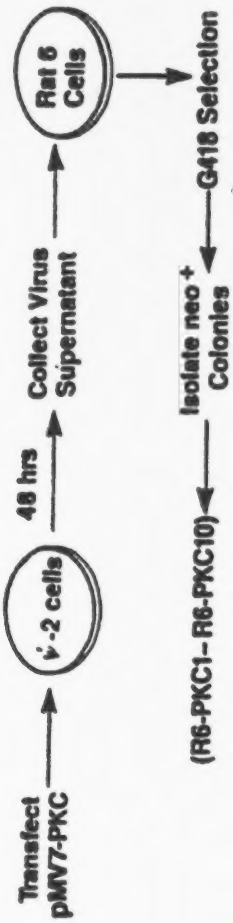

Transfect pMV7-PKC
ψ-2 cells
48 hrs
Collect Virus Supernatant
Rat 6 Cells
G418 Selection
Isolate neo+ Colonies
(R6-PKC1 – R6-PKC10)

FIG. 1.
C.

FIG. 2

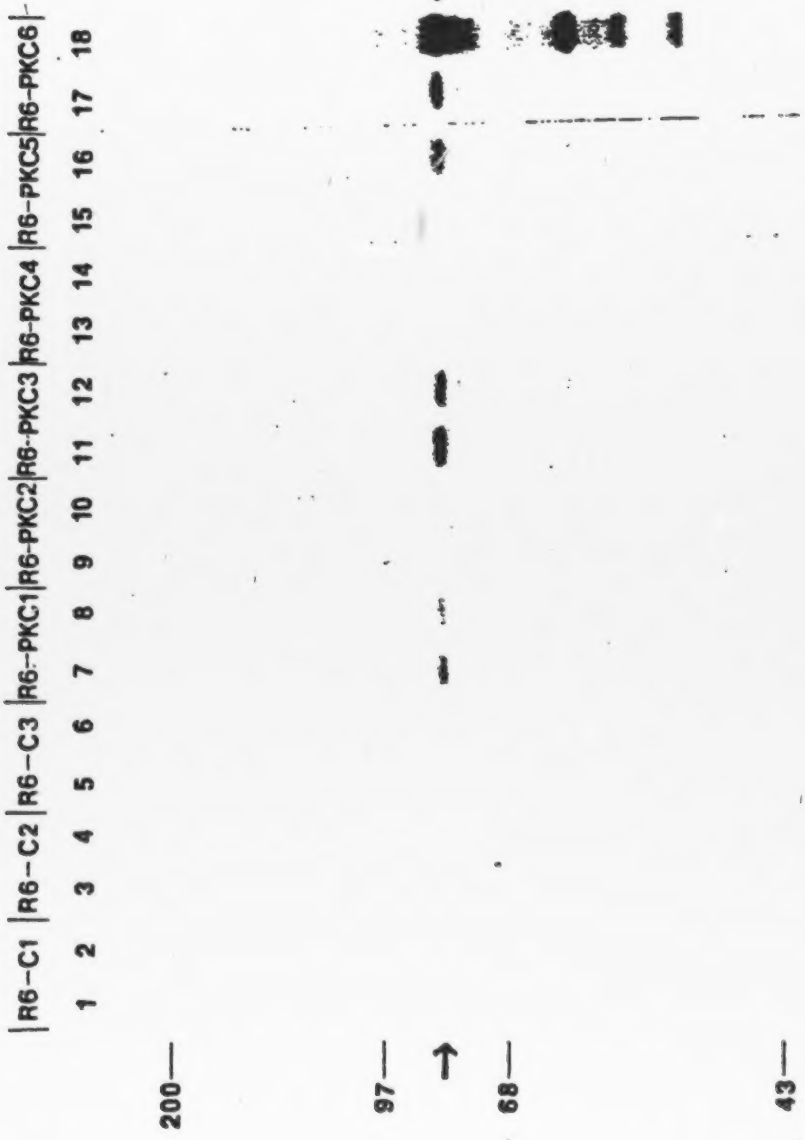

R6-C1
R6-C2
R6-C3
R6-PKC1
R6-PKC2
R6-PKC3
R6-PKC4
R6-PKC5
R6-PKC6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
200
97
68
43

FIG. 3

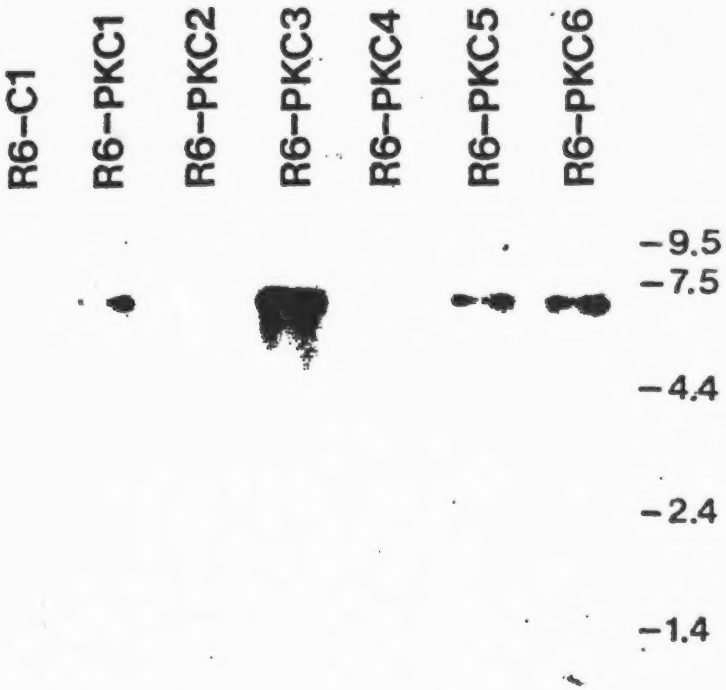
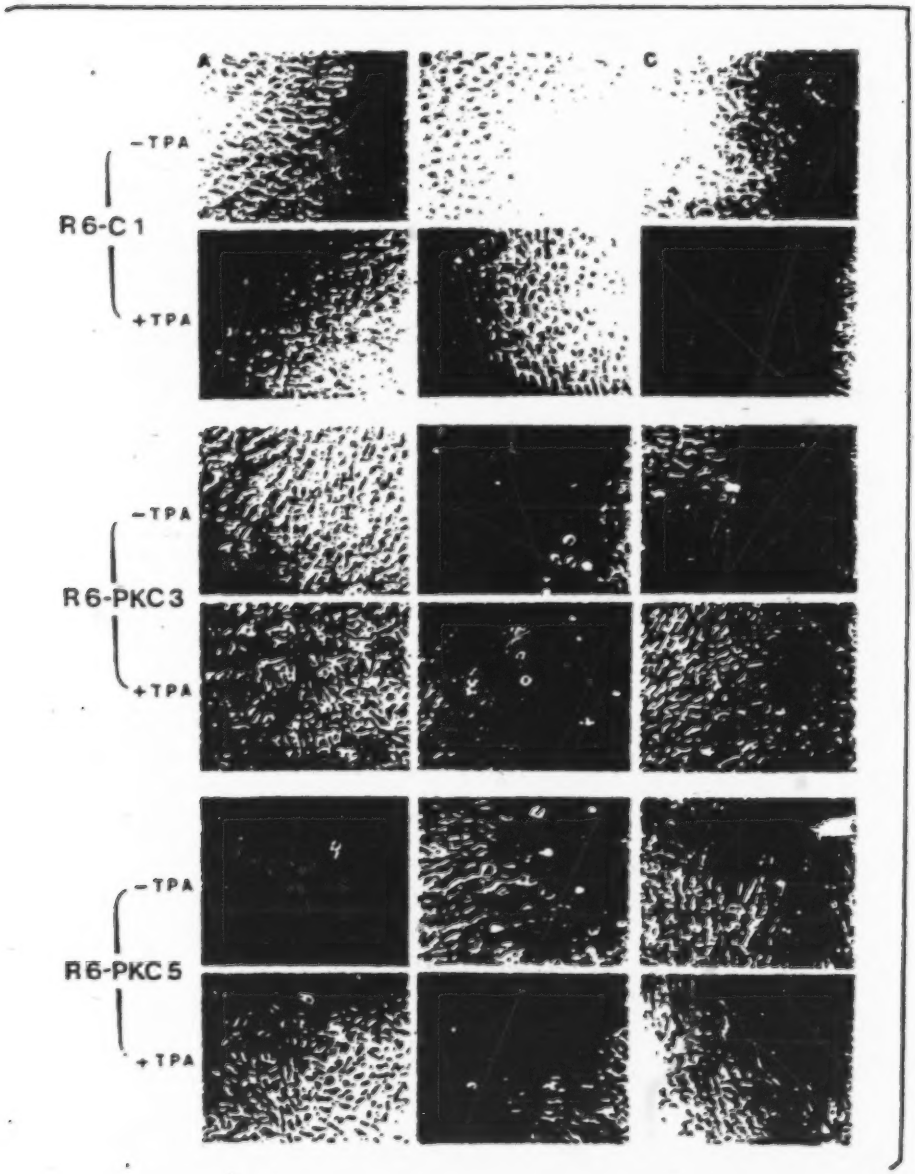

R6-C1
R6-PKC1
R6-PKC2
R6-PKC3
R6-PKC4
R6-PKC5
R6-PKC6
-9.5
-7.5
-4.4
-2.4
-1.4

FIG. 4



BEST AVAILABLE COPY

FIG. 5.

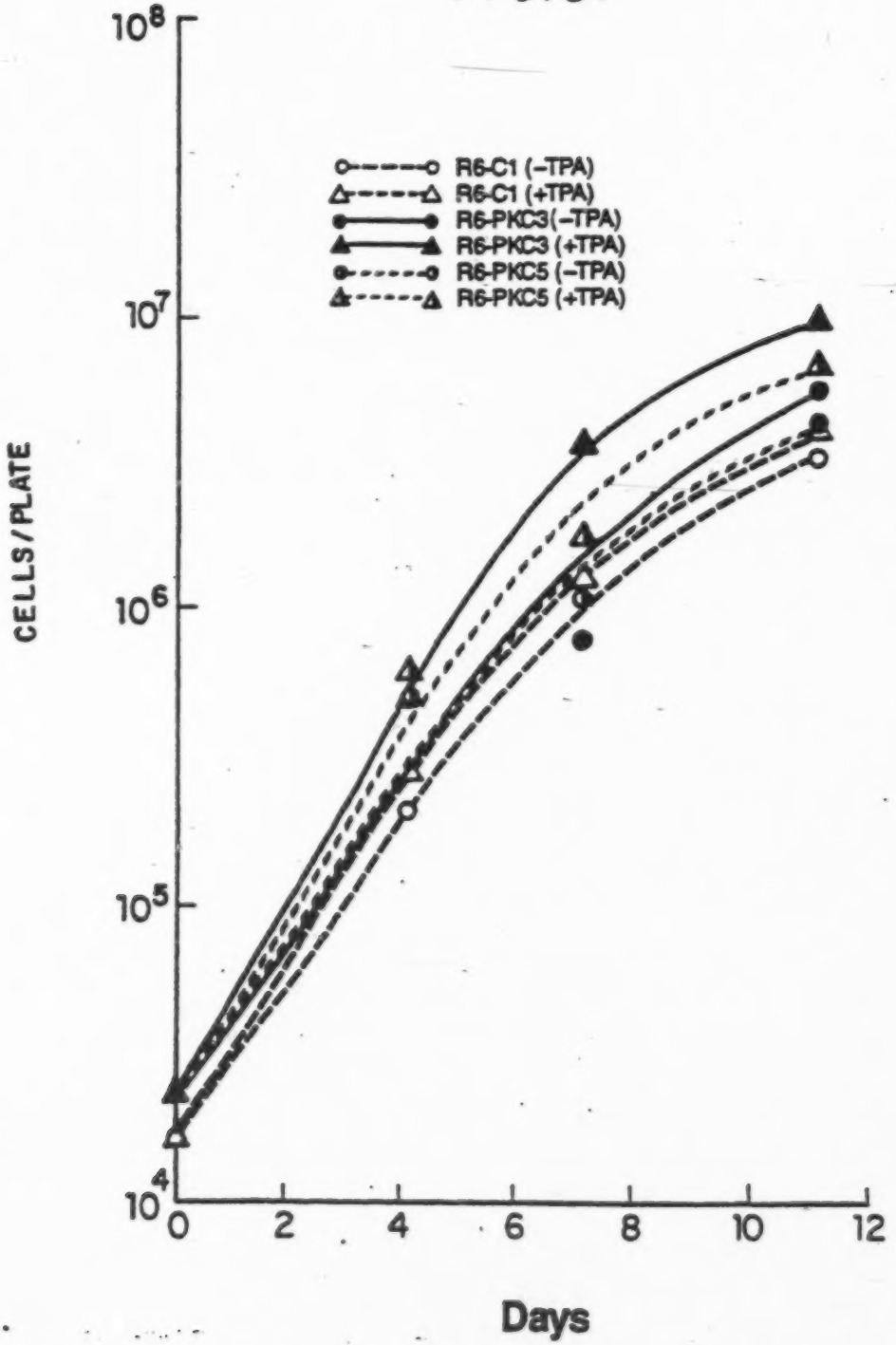

R6-C1 (-TPA)
R6-C1 (+TPA)
R6-PKC3(-TPA)
R6-PKC3 (+TPA)
R6-PKC5 (-TPA)
R6-PKC5 (+TPA)
CELLS/PLATE
10^8
10^7
10^6
10^5
10^4
0
2
4
6
8
10
12
Days

FIG. 6

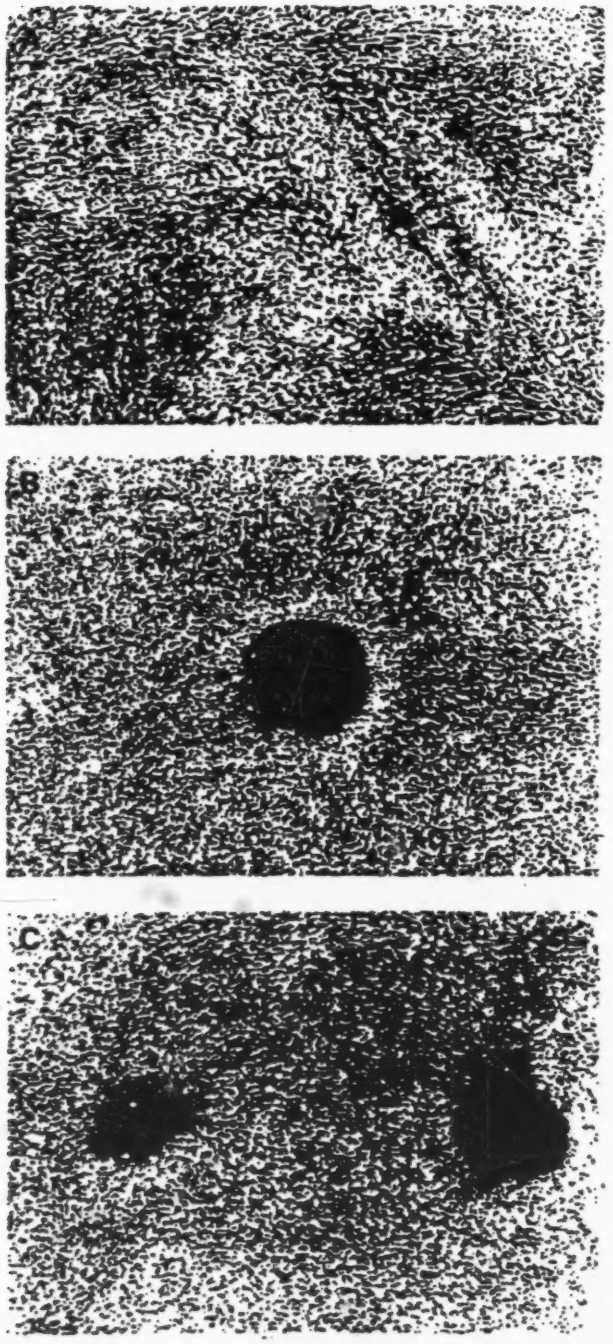


FIG. 7

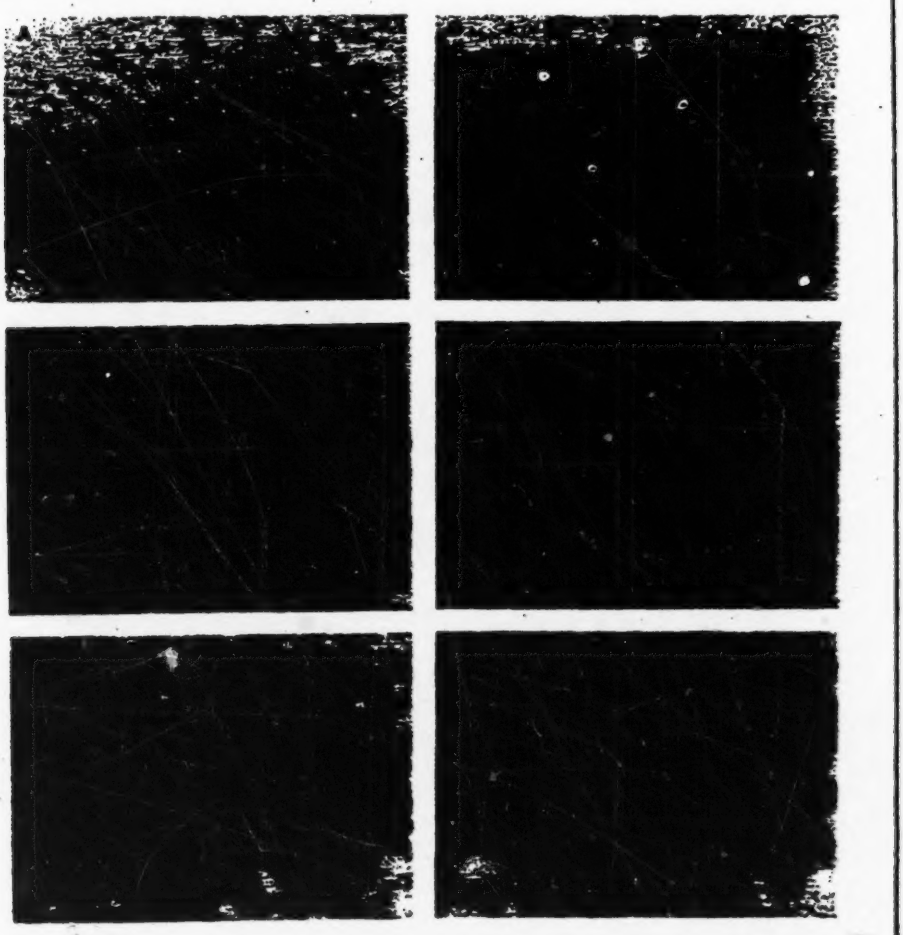


FIG. 1 CONT.

```
        2350        2360        2370        2380        2390        2400
         *           *           *           *           *           *
CAG AAA CTC ATC CAA TGA ACC AAC AGT GTC AAA ACT TAA CTG TGT CCG ATA CCA AAA TGC

        2410        2420        2430        2440        2450        2460
         *           *           *           *           *           *
TTC AGT ATT TGT AAT TTT TAA AGT CAG ATG CTG ATG TTC CTG GTC AAA GTT TTT ACA GTT

        2470        2480        2490        2500        2510        2520
         *           *           *           *           *           *
ACT CTC GAA TAT CTC CTT TGA ATG CTA CCT AAG CAT GAC CGG TAT TTT TAA AAG TTG TGA
```

METHOD OF SCREENING FOR PROTEIN INHIBITORS AND ACTIVATORS

This application is a continuation-in-part of U.S. Ser. No. 07/154,206, filed Feb. 10, 1988, now U.S. Pat. No. 4,980,281 incorporated by reference herein, the benefit of whose filing date is claimed pursuant to 35 U.S.C. Sec. 120.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a general screening method for the discovery and identification of both inhibitors and activators of enzymes, receptors, and other proteins. In particular, it is concerned with a method of screening for substances which specifically inhibit or activate a particular protein affecting the cultural or morphological characteristics of the cell expressing the protein, especially in a manner apparent to the naked eye.

2. Information Disclosure Statement

A number of assay systems are currently in use for the discovery of new modulators of cell growth, and in particular, in the search for new anti-cancer drugs which are specifically toxic to cancer cells but not to normal cells. A variety of changes may be scored for, but the most common ones are reversion of the transformed phenotype, significant changes in cell morphology, or cytotoxicity. The assays include: (1) in vitro cytotoxicity assays; (2) soft agar colony formation assays; (3) in vitro anti-microbial assays; and (4) assays which detect changes in cellular morphology.

In vitro cytotoxicity assays involve the measurement of cellular parameters which are indicative of inhibition of cellular growth or cytotoxicity. These include, for example, the measurement of the inhibition of certain cellular metabolic pathways in response to treatment with cytotoxic agents. The papers by Von Hoff, et al. (1985), and Catino, et al. (1985) describe typical methods which use this technique. These methods are somewhat complex technically, and require the use of radioactive tracers in some cases. Furthermore, the results are non-specific since any agent which alters the growth properties of cells will score positively in these assay systems.

Agents have also been tested for their ability to inhibit transformed (cancerous) cells from growing in soft agar. This method is based upon the finding by Freedman and Shin (1974) that the formation of colonies of cells in soft agar is the in vitro test which shows the highest correlation in predicting whether the cells will be tumorigenic in an experimental animal. This method is relatively simple to perform since colony growth will, after two or more weeks, generally be large enough to be seen with the naked eye. Scoring the final results, therefore, can be performed either by a technician without extensive training in tissue culture, or, as we describe in the current application, by an automated absorbence detection system. In its present form, however, this method is also non-specific for the same reasons as described above. In other words, any agent which inhibits cellular growth in any way will scores positively in this assay system as it is currently used, whether or not it inhibits the protein of interest.

In vitro anti-microbial assays involve the use of bacterial or yeast strains which are used as test organisms for screening for agents with generalized growth inhibitory properties (also described in Catino, et al., 1985). In this method, the bacterial or yeast strain is grown on standard media plates and potential agents are applied to various spots on the plates. If an agent has growth inhibitory properties, a clear zone results at the site of its application on the plate, resulting from the inability of the test strain to grow in the area. This method is rapid and can be performed by a technician without extensive training in tissue culture techniques, but the results are generally non-specific because agents which are effective against bacterial or yeast strains are frequently less effective (or completely ineffective) in modulating the growth of mammalian cells, as shown in the paper by Catino et al. (1985).

Still other screening systems depend upon a morphologic alteration of the test cells by exposure to the potential agents in order to determine the effectiveness of a given agent. This method is currently the most effective one for developing specific agents which interact with a given protein or alter a specific cellular property, as evidenced by the representative paper by Uehara, et. al. (1985). However, these screening systems are the most difficult ones to apply in practice, since the morphologic effect of each individual agent on the test cells must be studied under the microscope. Hence this method requires extensive observations of the cells by a trained scientist.

SUMMARY OF THE INVENTION

The Method presented in detail in this application combines the rapidity and ease of performance of the soft agar assay with a specificity for detecting an active agent exceeding that of the morphology assay. In brief, the method which we describe herein involves the generation of a cell line purposefully engineered to detect both stimulatory and inhibitory agents which are absolutely specific for any given protein which affects the cultural or morphological characteristics of the cell.

The basis for this invention is my observation that if a protein (the "protein of interest", or POI) which is involved in some manner in cellular growth control is overproduced in cells, then pharmacologic agents which can activate or inhibit the POI can result in altered growth properties of the cells.

The sensitivity of the cells is dependent on their production of the POI, a phenomenon referred to herein as a "graded cellular response" to the pharmacologically active agent.

The present invention provides a rapid, yet powerful screening system for the discovery and identification of both inhibitors and activators of proteins. The method may be applied to virtually any type of protein, including enzymes, receptors, DNA- or RNA-binding proteins, or others which are directly or indirectly involved in regulating cellular growth.

The method involves the insertion of a DNA (or cDNA) sequence encoding the Protein Of Interest (POI) into an appropriate vector and the generation of cell lines which contain either (1) the expression vector alone ("control" cell lines) or (2) the expression vector containing the inserted DNA (or cDNA) sequence encoding the POI ("test" cell lines). Using the appropriate vector system, recipient cell lines, and growth conditions, test cell lines can thus be generated which stably overproduce the corresponding POI. Under the appropriate growth conditions, these cell lines will exhibit a "graded cellular response" to activators or inhibitors of the POI. A screening system can thus be set up whereby

the control and test cell lines are propagated in defined growth conditions in tissue culture dishes (or even in experimental animals) and large numbers of compounds (or crude substances which may contain active compounds) can be screened for their effects on the POI.

Substances which inhibit or activate the POI may affect characteristics such as growth rate, tumorigenic potential, anti-tumorigenic potential, anti-metastatic potential, cell morphology, antigen expression, and/or anchorage-independent growth capability. Substances which specifically inhibit or inactivate the POI may be distinguished from substances which affect cell morphology or growth by other mechanisms in that they will have a greater effect on the test lines than on the control lines.

The system has been tested using several cDNA sequences and several recipient cell lines, and can be easily automated.

The appended claims are hereby incorporated by reference as an enumeration of the preferred embodiments. All references cited anywhere in this specification are hereby incorporated by reference to the extent pertinent.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows the full-length cDNA sequence, and the deduced amino acid sequence, of one of several forms of PKC which has previously been isolated (cDNA clone RP58), and whose partial sequence has been reported (Housey, et al., 1987). It corresponds to PKCbetaI according to the nomenclature of Ono, et al. (1987). The deduced amino acid sequence begins with the first in-frame methionine codon at nucleotide position 91 and encodes a 671 amino acid protein with a predicted molecular weight of 76.8 kd. A consensus polyadenylation signal is underlined.

FIG. 1B shows the retrovirus-derived cDNA expression vector, developed in this laboratory, which was used for the present studies. The full-length RP58 cDNA encoding PKC betaI (shown in IA) was cloned into the Eco RI site of plasmid pMV7. The shaded region represents the coding sequence. "E" and "P" designate Eco RI and Pst I restriction sites, respectively. The indicated sizes between restriction sites in the RP58 cDNA are given in kilobases. "LTR" designates the 5' (left) and 3' (right) long terminal repeats of Moloney murine leukemia virus, and "TK-neo" designates the promoter region of the HSV thymidine kinase gene linked to the 5' end of the bacterial neomycin phosphotransferase (neo) gene.

FIG. 1-C outlines in schematic form the overall strategy used to generate cell lines stably overproducing PKC.

FIG. 2. Purification and Autophosphorylation of PKC. PKC activity from each cell line was purified and subjected to reaction conditions favoring autophosphorylation of PKC. Following the autophosphorylation reaction, protein samples were separated by discontinuous polyacrylamide gel electrophoresis. In the lanes bearing odd numbers the reaction mixtures contained 1 mM Ca2+ and phosphatidylserine to activate PKC, and in the lanes bearing even numbers the reaction mixtures contained 1 mM EGTA, 100 ng/ml TPA, and phosphatidylserine. The numbers in the left margin indicate the sizes of molecular weight markers, in kd. Arrows indicate the position of the 75 kd autophosphorylated PKC.

FIG. 3. Northern Blot Hybridization Analyses. Poly A+ RNA was isolated from the indicated cell lines and separated by electrophoresis on 6% formaldehyde/1% agarose gels, blotted onto nylon membrane and hybridized with the 32P-labelled full-length RP58 cDNA probe, as previously described (Housey, et. al., 1987). The numbers in the right margin indicate the sizes in kb of the RNA markers. The R6-PKC4 sample displayed a very weak 4.8 kb band on the original autoradiograph.

FIG. 4. Morphologic Responses of the Cell Lines to Phorbol Ester Treatment. Nearly confluent cultures of the three indicated cell lines were exposed to 100 ng/ml TPA in 0.1% dimethylsulfoxide (DMSO) solvent ("+TPA") or 0.1% DMSO alone ("-TPA"), in DMEM plus 10% CS. Photographs were taken 24 hours later (Panels A and D) and 48 hours later (Panels B and E). Fresh medium plus or minus TPA was then added and photographs were then taken an additional 24 hours later (Panels C and F). (Magnification: 100X).

FIG. 5. Growth Curves of Control and PKC Overproducing Cell Lines. The indicated cell lines were seeded at 1×10^4 per 6 cm plate in DMEM plus 10% CS, in the presence ("+TPA") and absence ("-TPA") of 100 ng/ml TPA. Cell numbers were determined in replicate plates during the subsequent 11 day growth period. The values given indicate the means of triplicate determinations, which varied by less than 10%.

FIG. 6. Post-confluence Foci Formation. Control R6-C2 cells (Panel A) and R6-PKC3 cells (Panels B and C) were grown to confluence and then maintained for an additional 28 days in DMEM plus 10% CS (without TPA) with the addition of fresh medium every 3 days. Photographs were taken at the end of the 28 day period. Magnification: 40×.

FIG. 7. Growth in Soft Agar. Cell lines R6-C1, R6-PKC3, and R6-PKC5 were seeded into 60 mm petri dishes in 0.3% agar containing DMEM plus 20% FBS and 50 lg/ml G418, plus or minus 100 ng/ml TPA. Photographs were taken after 21 days of growth.A) R6-C1+TPA (low-power field) B) R6-C1+TPA (medium-power field)C) R6-PKC3 D) R6-PKC3+TPA E) R6-PKC5 F) R6-PKC5 +TPA For additional details see Experimental Procedures. (Magnification: 100× in panel B: 40× in all other panels).

DETAILED DESCRIPTION OF THE INVENTION

The present method is intended for use in identifying potential chemical inhibitors or activators of enzymes, receptors, or any proteins which have effects upon cell phenotype. This method requires two cell lines, preferably alike except for their expression (production) of the protein of interest at different levels (and any further differences necessitated by that difference in expression). Inhibitors or activators are identified by their greater effect on the phenotype of the higher producing cell line.

Any phenotypic characteristic of the cell which is affected by expression of the protein of interest, other, of course, than the level of the protein itself, may be assayed. The phenotypic characteristic is preferably a "cultural" or "morphological" characteristic of the cell. For purposes of the appended claims, these terms are defined as follows:

Cultural characteristics include, but are not limited to the nutrients required for growth, the nutrients which, though not required for growth, markedly promote growth, the physical conditions (temperature, pH, gase-

ous environment, osmotic state, and anchorage dependence or independence) of the culture which affect growth, and the substances which inhibit growth or even kill the cells.

Morphological characteristics, but are not limited to include the size and shape of cells, their arrangements, cell differentiation, and subcellular structures.

Where the protein of interest is implicated in tumorigenesis or related phenomena,, the characteristic observed is preferably one related to cellular growth control, differentiation, de-differentiation, carcinogenic transformation, metastasis, tumorigenesis, or angiogenesis.

Phenotypic changes which are observable with the naked eye are of special interest. Changes in the ability of the cells to grow in an anchorage-independent manner, to grow in soft agar, to form foci in cell culture, and to take up selected stains are particularly appropriate phenomens for observation and comparison.

The higher producing cell line is preferably obtained by introducing a gene encoding the Protein of Interest (POI) into a host cell. The gene may be a one isolated from the genome of an organism, a cDNA prepared from an mRNA transcript isolated from an organism, or a synthetic duplicate of a naturally occurring gene. It may also have a sequence which does not occur exactly in nature, but rather corresponds to a mutation (single or multiple) of a naturally occurring sequence. No limitation is intended on the manner in which this mutated sequence is obtained. The gene is operably linked to a promoter of gene expression which is functional in the host, such that the corresponding Protein Of Interest (POI) is stably "overproduced" in the recipient cells to differing degrees. The promoter may be constitutive or inducible. By "overproduced", I mean that the POI is expressed at higher levels in the genetically manipulated cell line than in the original cell line. This allows one to generate cell lines which contain (or secrete) from as little as a few fold to more than 100-fold elevated levels of the POI relative to the control cells.

Any method may be used to introduce the gene into the host cell, including transfection with a retroviral vector, direct transfection (e.g., mediated by calcium phosphate or DEAE-dextran), and electroporation. Preferably, a retroviral vector is used.

The host cells should exhibit a readily observable phenotypic change as a result of enhanced production of the POI. Preferably, this response should be proportional to the level of production of the POI. Finally, the cells should not spontaneously manifest the desired phenotypic change. For example, 3T3 cells form foci spontaneously. Among the preferred cell lines are Rat-6 fibroblasts, C3H10T1/2 fibroblasts, and HL60. (HL60 is a human cell line that differentiates in response to PKC activation.) 3T3 cells may be used, but with the reservation stated above.

Generally speaking, it is preferable to maximize the ratio of production by the "overproducing" cell line to production by the "native" line. This is facilitated by selecting a host cell line which produces little or no POI, and introducing multiple gene copies and/or using high signal strength promoters.

The Rat 6 embryo fibroblast cell line is a variant of the rat embryo fibroblast cell line established by Freeman et. al., (1972) and isolated by Hsiao et al., 1986. This cell line has an unusually flat morphology, even when maintained in culture at post-confluence for extended periods of time, displays anchorage dependent growth and, thus far, has not undergone spontaneous transformation. It was also ideal for these studies since it has a very low level of endogenous PKC activity and a low level of high affinity receptors for phorbol esters.

While my most preferred host cell line is the Rat-6 fibroblast cell line, I have tested this Method with other cell types, including the mouse NIH-3T3 cell line as well as the C3H 10T1/2 cell line. Tables 1(a) and (b) below show the representative specific activities of seven NIH-3T3 and six C3H-10T1/2 cell lines stably overproducing PKC. I have also recently performed the same experiments with the human HeLa cell line. In each case the resulting cell lines all exhibited growth properties qualitatively identical to those described for the PKC-expressing Rat-6 fibroblast cell lines. More specifically, C3H 10T1/2 and NIH 3T3 cells which overproduced PKC exhibited increased cell density, refractility, and rounding relative to the "native" lines. The PKC-overproducing NIH 3T3 cells also displayed increased foci formation. PKC-overproducing C3H 10T1/2 cells also formed foci, but only when exposed to a phorbol ester, i.e., a PKC activator. These results clearly demonstrate that many different types of cells can be employed in this method. The experimental procedures used to generate these cell lines were also identical to those used in connection with the Rat-6 cell line.

If a cell line otherwise suitable for use as a control cell line produces excessive POI, it is possible to inhibit this production by incorporation a known inhibitor into the culture medium for both the control and test cell lines, thus achieving a more favorable ratio of production. Contrawise, if the level of POI production by the test cell line is too low, a known activator may be incorporated into the culture media.

It is desirable, but not necessary, that a suspected inhibitor or activator be tested on both a control line and an overproducing line in parallel.

What we are looking for is a increase in the phenotypic change exhibited by the cell which becomes greater with increasing expression of the POI. We call this a "graded cellular response," and it is by this specialized response that we distinguish inhibitors or activators of the POI from agents that act upon other cell metabolites to effect a phenotypic change.

Thus, in a preferred embodiment, the cell lines are assayed for their relative levels of the POI, and their ability to grow in anchorage-independent systems (e.g., matrices such as soft agar or methocel), to form small "foci" (areas of dense groups of cells clustered together and growing on top of one another) in tissue culture dishes, to take up selected stains, or to bind an appropriately labeled antibody or other receptor for a cell surface epitope. In addition to exhibiting these growth control abnormalities, such cell lines will also be sensitive in their growth properties to chemical agents which are capable of binding to, or modifying the biological effects of, the POI.

The method is particularly unique in that it can be employed to search rapidly for EITHER activators OR inhibitors of a given POI, depending upon the need. The term "activators," as used herein, includes both substances necessary for the POI to become active in the first place, and substance which merely accentuate its activity. The term "inhibitors" includes both substance which reduce the activity of the POI and these which nullify it altogether. When a POI has more than one possible activity. The inhibitor or activator may modulate any or all of its activities.

The use of this screening method to identify inhibitors or activators of enzymes is of special interest. In particular, I am interested in using it to identify inhibitors or activators of enzymes involved in tumorigenesis and related phenomena, for example, protein kinase C, ornithine decarboxylase, cyclic AMP-dependent protein kinase, the protein kinase domains of the insulin and EGF receptors, and the enzyme products of various cellular onc genes such as the c-src (PP^{60}src) or c-ras ($P20^{ras}$) genes.

The present invention may be used to identify activators or inhibitors of receptor proteins, both cytoplasmic receptors and integral membrane receptors, provided that overproduction of the receptor protein has a detectable effect on the producing cells.

Protein kinase C (PKC) is a Ca^{2+}— and phospholipid-dependent serine/threonine protein kinase of fundamental importance in cellular growth control. PKC is activated endogenously by a wide variety of growth factors, hormones, and neurotransmitters, and has been shown to be a high affinity receptor for the phorbol ester tumor promoters as well as other agents which possess tumor promoting activity (for reviews see Nishizuka 1986; 1984; Ashendel, 1984). PKC has been shown to phosphorylate several intracellular protein substrates, including the epidermal growth factor (EGF) receptor (Hunter et al., 1984), pp60src (Gould et al., 1985), the insulin receptor (Bollag et al., 1986), p21 ras (Jeng et al., 1987), and many others (Nishizuka, 1986). Several laboratories have recently isolated cDNA clones encoding distinct forms of PKC, thus demonstrating that PKC is encoded by a multigene family (Ono et al., 1986, Knopf et al., 1986, Parker et al., 1986; Coussens et al., 1986; Makowske et al., 1986; Ohno et al., 1987; Housey et al., 1987). The multiple forms of PKC exhibit considerable tissue specificity (Knopf, et. al., 1986; Brandt et al., 1987; Ohno, et al, 1987; Housey, et. al., 1987) which suggests that there may be subtle differences in the function(s) of each of the distinct forms. However, all of the cDNA clones which have been isolated thus far that encode distinct forms of PKC share at least 65% overall deduced homology at the amino acid level, and transient expression experiments with some of these cDNA clones have shown that they encode serine/threonine protein kinase activities which bind to, or are activated by, the phorbol ester tumor promoters (Knopf, et. al., 1986, Ono, et. al., 1987).

We used the PKCbetaI cDNA clone for the present studies for the following reasons. With the exception of the brain, where its expression is very high, PKCbetaI is expressed at very low levels in most tissues, and its expression is virtually undetectable in Rat 6 fibroblasts (see below). Thus, we reasoned that using this form would maximize the phenotypic differences observed between control cells and cells overexpressing the introduced form of PKC. The PKCbetaI form is also of particular interest because within the PKC gene family its deduced carboxyl terminal domain displays the highest overall homology to the catalytic subunit of the cyclic AMP-dependent protein kinase (PKAc) and the cyclic GMP-dependent protein kinase (PKG) (Housey et al., 1987). The latter observation suggests that PKAc, PKG, and the betaI form of PKC may share a common ancestral serine/threonine protein kinase progenitor, and that the additional PKC genes may have been derived through evolutionary divergence from the betaI form.

Agents which interact with certain structural proteins, such as actin and myosin, are also of interest. Mutations in the genes expressing these proteins may be involved in tumorigenesis and metastasization. Such interactions can lead to changes in cell phenotype which can be assayed by this method.

As is set forth in greater detail below, I have produced cell lines which overproduce protein kinase C (PKC). These cell lines, unlike the control cells, grow in soft agar even in the absence of the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA has been shown to be a potent activator of PKC. When TPA is added to the growth medium the PKC-overproducing cell lines grow even better and form considerably larger colonies in soft agar. Furthermore, I have also tested known inhibitors of PKC activity which, as predicted, caused the PKC-overproducing cells to grow less well (or not at all) in soft agar. Thus, the direct utility of this method in identifying both activators or inhibitors of a gene product, in this case PKC, has been clearly demonstrated.

In additional studies with other genes, most notably the c-H-ras oncogene, the c-myc oncogene, and certain cDNA clones encoding phorbol-ester inducible proteins, overproducing cell lines which exhibited morphological changes relative to the native lines were likewise obtained, and it is believed that such cell lines may be used in screening for inhibitors and activators of the overproduced proteins. Thus it is clear that the method can be generalized to a wide variety of genes encoding proteins which are involved in cellular growth control in numerous cell types. These studies are described in more detail below.

First I tested the capability of pMV7-based expression vectors (pMV7 is my preferred transfer vector) to produce several different types of proteins in various cell lines. I used the cDNA sequences encoding the following proteins: hypoxanthine/guanine phosphoribosyltransferase (HGPRT), the human T4 lymphocyte cell surface antigen, the human T8 lymphocyte cell surface antigen, and ornithine decarboxylase (ODC). In each case the pMV7 vector was capable of producing high levels of expression of the relevant gene thereby resulting in overproduction of the corresponding protein product.

Once I had verified that the pMV7-derived expression vector could reproducibly generate cell lines which stably overproduced proteins I then tested additional genes which encode proteins, other than PKC, which are also involved in cellular growth control.

In the first case I chose a cDNA clone which, in collaboration with others, I had previously isolated and characterized. This cDNA clone, designated TPA-S1, encodes a protein of as yet unknown function. Thus I chose this clone for the express purpose of testing the method under conditions where a gene is used which encodes a protein of unknown function. Since we had previously demonstrated that the transcription of the TPA-S1 gene is rapidly and strongly induced following the treatment of cells with tumor promoters such as TPA (Johnson, et al., 1987), it appeared that the TPA-S1 gene product played some role in cellular growth control, but we had no additional data regarding its function. Thus, to further test the Method, the TPA-S1 cDNA was cloned into the pMV7 expression vector, resulting in a plasmid construct designated pMV7-TPA-S1, and cell lines were generated which stably overexpressed the TPA-S1 cDNA clone. These cell

lines were generated exactly as described for the R6-PKCx series cell lines described below.

In this case the results were also qualitatively identical since the NIH/3T3-tpa-s1 cell lines which were generated that stably overproduced the tpa-s1 encoded protein were also found to exhibit anchorage independent growth in soft agar. This growth, furthermore, was dependent upon the level of TPA-S1 mRNA and TPA-S1 protein which was being synthesized in each cell line. Thus these cell lines could be used, in strictly analogous manner to the R6-PKCx series cell lines, for the development of either an inhibitor or an activator of the TPA-S1 protein using the Method as described herein. It should be noted, in addition, that in these experiments we were able to obtain cell lines overproducing TPA-S1 which exhibited plating efficiencies in soft agar in excess of 80%, even though the TPA-S1 gene does not appear to be an oncogene by the standard definition of the latter term.

In further experiments, I have tested the method using an activated c-H-ras oncogene (T24), again in analogous fashion to the techniques described herein, and again with analogous results to those described herein for both the PKC cDNA clone and the TPA-S1 cDNA clone. Thus, the Method can also be used for the rapid development of a p21 ras inhibitor. Taken together, the results described in this application demonstrate directly that the Method described herein is clearly generalizable to any gene which is involved in any way in cellular growth control.

In further studies, we have generated additional cell lines which stably overproduce other proteins-of-interest (POIs). In one set of studies, we have introduced the betal form of protein kinase C (PKC) into a bone-marrow derived cell line, FDC-P1, and demonstrated that the resulting cell lines are profoundly affected by PKC activators. In a second set of studies, we have developed a screening assay system for insulin agonists and antagonists which utilizes cell lines which stably overproduce the insulin receptor. In these additional studies we have utilized different cell lines, expression vector systems, cDNAs encoding other POIs, and in general varied all of the major parameters of our initial studies in order to demonstrate the true generalizability of the system to any type of cell and any protein-of-interest.

The preferred protein inhibitor/activator drug screening method of the present invention comprises the following steps:

1. Construction of an expression vector which is capable of expressing the protein of interest in the selected host by inserting a gene encoding that protein into a transfer vector. The gene may be inserted 3' of a promoter already borne by the transfer vector, or a gene and a promoter may be inserted sequentially or simultaneously.

2. Introduction of the expression vector (a) into cells which produce recombinant retrovirus particles, or (b) directly into host cells which will be used for subsequent drug screening tests (the resulting cells are called herein "test" cells).

In parallel, the transfer vector (i.e., the vector lacking the gene of interest and possibly a linked promoter but otherwise identical to the expression vector is preferably also introduced into the host cells. Cell lines derived from this latter case will be used as negative controls in the subsequent drug screening tests. Alternatively, the unmodified host cells may be used as controls.

If 2a was employed, after an appropriate time (usually 48 hours), media containing recombinant virus particles is transferred onto host cells so as to obtain test or control cells.

3. The test and control cells are transferred to selective growth medium containing the appropriate drug which will only allow those cells harboring the expression vector containing the selectable marker gene (as well as the gene or cDNA of experimental interest) to grow. After an appropriate selection time (usually 7–10 days), individual clones of cells (derivative cell lines) are isolated and propagated separately.

4. Each independent cell line is tested for the level of production of the POI. By this method, a range of cell lines is generated which overproduce from a few fold to more that 100-fold levels of the POI. In parallel, the control cell lines which contain only the transfer vector alone (with the selectable marker gene) are also assayed for their endogenous levels of the POI.

5. Each independent line is then tested for its growth capability in soft agar (or methocel, or any other similar matrix) of various percentages and containing different types of growth media until cell lines are identified which possess the desired growth characteristics as compared to the control cell lines.

6. Each cell line is also tested for its ability to form "foci", or areas of dense cellular growth, in tissue culture plates using media containing various percentages and types of serum (20%, 10%, 5% serum, fetal calf serum, calf serum, horse serum, etc.) and under various conditions of growth (e.g. addition of other hormones, growth factors, or other growth supplements to the medium, temperature and humidity variations, etc.). In these tests, the cells are maintained at post-confluence for extended periods of time (from two to eight weeks) with media changes every three days or as required. Such growth parameters are varied until cell lines are identified which possess the desired foci formation capacity relative to the control cell lines under the identical conditions.

7. After a cell line possessing the required growth characteristics is identified, the cells are grown under the conditions determined in (5) above with the growth medium supplemented with either crude or purified substances which may contain biologically active agents specific to the POI. Thus, crude or purified substances possessing the latter properties can be rapidly identified by their ability to differentially alter the growth properties of the experimental cells (which overproduce the POI) relative to the control cells (which do not). This can be done rapidly even by simple observation with the naked eye, since the colonies which grow in soft agar after 2 weeks are easily seen even without staining, although they may be stained for easier detection.

Similarly, if the post-confluence foci formation assay is chosen, the foci which result after approximately two weeks can be easily seen with the naked eye, or these foci can also be stained. For screening very large numbers of compounds (tens of thousands or more), the entire procedure can be performed on 96 well tissue culture plates. This applies equally well for either the soft agar growth assay or the tissue culture foci formation assay. Results of the assays can be rapidly determined by measuring the relative absorbance of the test cells as compared to the control cells (at 500 nm, or another appropriate wavelength). Absorbance readings may be rapidly performed in a 96-well plate absorbance

reader such as the "Titer-tek" plate reader, or any of several analogous apparatus currently available. In this fashion, thousands of compounds could be screened per month for their biological activity with very low labor and materials costs.

Furthermore, if antigen expression varies on the test cells expressing high levels of the POI relative to the control cells, a simple Enzyme-Linked Immunoadsorption Assay (ELISA) could be performed and an antibody specific to the antigen.

While the assay may be performed with one control cell line and one test cell line, it is possible to use additional lines, tests lines with differing POI levels. Also additional sets of control/test lines, originating from other hosts, may be tested.

Specific examples implementing the series of steps described above are as follows.

EXAMPLE 1

If one were interested in screening for a protein kinase C (PKC) inhibitor, cell lines would be generated and selected which grow well in soft agar (as a result of their overproduction of any form of PKC) and yet show an enhancement of their growth when compounds which are known to stimulate PKC are added to the growth medium. Appropriate control cells, of course, would not exhibit any of these characteristics. Screening for a potent PKC inhibitor could then be performed by searching for those substances which could selectively inhibit the soft agar (anchorage-independent) growth of the PKC-overproducing cell lines. Alternatively, since the PKC-overproducing cells also form small, dense foci in tissue culture, one could also screen for substances which inhibit this foci formation.

Described below are the detailed aspects of the relevant techniques and methods used to apply the principles of the invention to the problem of developing a system useful for screening for potent inhibitors of protein kinase C (PKC), a high-affinity intracellular receptor for tumor-promoting agents. The cell lines which resulted from the application of this method are highly sensitive and responsive both to agents which activate PKC as well as to those which inhibit PKC.

Construction of plasmid pMV7

The construction of pMV7 was begun with plasmid pPyori which contains the polyoma virus origin of replication cloned into the unique BamHI site of pML-1 (Lusky and Botchan, 1981). This plasmid replicates in murine cells that contain the polyoma T antigen (Dailey and Basilico, 1985). Plasmid pMV (Perkins et al., 1983) was then cleaved with HincII and BglI. The 2.29 kb fragment that contains the Moloney Leukemia Virus Long Terminal Repeats (LTR), the packaging site, the splice donor site, and the proline tRNA binding site was isolated.

During the original construction of pMV (Perkins et al., 1983) 3.95 kb was removed from the MSV genome by cleaving with PstI. This left a PstI site situated 380 bps 3' from the tRNA binding site and 308 bps from the 540 bps end of the 3' LTR (Reddy et al., 1981). Xho I linkers were added to the HincII-BglI fragment, and to pPyori after it had been cleaved with EcoRI and HindIII. The two fragments were ligated, after activation of the linkers, and a plasmid, designated pMV-3, that contained the Moloney Virus control elements was isolated.

The unique EcoRI site was removed from this vector by digesting the plasmid with EcoRI and treating the linear molecules with T4 polymerase. These molecules were circularized and a plasmid, pMV-4, lacking the EcoRI site, was isolated. An EcoRI linker was inserted into this plasmid at the PstI site between the Moloney LTRs; the resultant plasmid was designated pMV-5.

The dominant selectable marker (neo) was added to pMV-5. The first step was isolating a 1.9 kb BamHI-SalI fragment from pIPBl. This fragment contains the Herpes Simplex virus thymidine kinase (tk) promoter region and the coding sequence for the bacterial neo gene (neomycin phosphotransferase). This fragment was blunt-ended with T4 polymerase, ClaI linkers were added and the fragment was cloned into the Cla I site 165 bps 3' to the EcoRI site in pMV-5, between the LTRs. This plasmid was designated pMV5-tk neo. The tk promoter has an EcoRI site 70 bps 5' to the start of transcription. This EcoRI site was removed by partially digesting pMV5-tkneo with EcoRI, isolating the linear full length cut species, filling in the ends with T4 polymerase and recircularizing the molecule. A plasmid was chosen in which the EcoRI site previously present in the tk promoter sequence was removed, but the EcoRI site 537 bps 3' to the start of transcription of the 5' LTR was retained. This plasmid was designated pMV7 and a map of this plasmid is shown in FIG. 1B.

This vector consists of the 5' and 3' Moloney Murine Leukemia Virus (MoMuLV) LTRs, the MoMuLV RNA packaging site 3' to the 5' LTR, an Eco RI cloning site, a modified Herpes Simplex Virus thymidine kinase (tk) promoter (lacking an Eco RI site at −79 bp), and the selectable marker gene neo. cDNA clones inserted into the EcoRI cloning site are under the transcriptional control of the 5' LTR, whereas the neo gene is independently transcribed by the tk promoter. This structure favors maintenance of the functional integrity of the selectable marker without interfering with the expression of the 5' (unselected) cDNA sequence. It is known (Maddon et al., 1986; Daley et al., 1987) that when various cDNA sequences are inserted into the EcoRI site of pMV7 they can be readily transferred into recipient cells by virus-mediated passage, are stably expressed, and yield high-level production of the corresponding protein.

Nucleotide Sequencing and Expression Vector Construction

Nucleotide sequencing of the PKC cDNA clone RP58, a full-length clone isolated from a rat brain cDNA library, which corresponds to the previously reported clone RP41, was performed as previously described (Housey et al., 1987). The full-length cDNA sequence of RP58, which encodes PKCbetaI (FIG. 1A), was subcloned into the EcoRI site of plasmid pMV7 using standard methodology (Maniatis et al., 1983). The general structure of pMV7 is shown in FIG. 1B. The construct resulting from insertion of the PKCbetaI gene is designated pMV7-PKCbetaI.

Isolation of cell lines stably overexpressing PKC

20 ug of CsCl banded pMV7 or pMV7-PKCbetaI plasmid DNA were transfected (Graham and van der Eb, 1973; as modified by Wigler et al., 1977) onto subconfluent "Psi-2" cells (Mann et al., 1985). After 48 hrs the culture medium was collected, filtered through a 0.45 um filter and stored at −70° C. Recipient subconfluent Rat-6 fibroblasts (5×10^5 per 10 cm plate) were

infected with the virus-containing medium in 2 ug/ml polybrene for 48 hrs. The cells, grown to confluence, were then trypsinized and replaced in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (CS) (Flow Laboratories) with 200 ug/ml of the neomycin derivative G418 (Geneticin). Resistant colonies were cloned by ring isolation after 1 week of G418 selection.

After approximately ten days of growth in selective medium, ten individual G418-resistant clones were isolated and maintained independently in G418-containing medium. These lines were designated R6-PKCbetall through R6-PKCbeta110 (abbreviated as R6-PKC1 through R6-PKC10). In parallel, a set of control Rat 6 lines was generated by transfection of the plasmid pMV7 (lacking the PKC cDNA insert) onto W-2 cells, infection of recipient Rat-6 cells, and selection for G418 resistance as described above for plasmid pMV7-PKCbetal. Similarly, after ten days of growth in the G418-containing medium, five individual, well-isolated G418-resistant clones were then isolated and maintained independently. These control lines were designated R6-C1 through R6-C5.

RNA Isolation and Blot Hybridization Analyses

Poly A+ RNA isolations, gel electrophoresis, and blot hybridization analyses were performed as previously described (Housey, et. al., 1985). RNA molecular weight markers were obtained from Bethesda Research Laboratories. The 2.7kb cDNA insert of RP58 (see above) was subcloned into plasmid pKS(+) (Stratagene Cloning Systems) to yield a plasmid designated pS2-RP58. A ^{32}P-labelled probe was prepared from pS2-RP58 and used under high-stringency hybridization conditions as previously described (Housey et al., 1987).

Purification and Assay of PKC Activity From Tissue Culture Cells

The total PKC activity (membrane-associated plus cytosolic) present in cultured cells was determined after partial purification of cellular extracts as follows.

Three 10 cm plates of confluent cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS) and then 10 ml of homogenization buffer (20 mM Tris, pH 7.5, 5 mM EGTA, 5mM EDTA, 15 mM 2-mercaptoethanol, 10 ug/ml soybean trypsin inhibitor, 10 ug/ml leupeptin, 40 ug/ml phenylmethylsulfonyl fluoride), containing 0.1% Triton X-100 were added. The cells were then scraped from each of the plates, pooled and disrupted with 25 strokes in a Dounce homogenizer. The homogenate was transferred to a 15 ml disposable polystyrene tube, centrifuged at 2000×g for 5 minutes at 4° C., and the supernatant was loaded onto a 1 ml DEAE Sephacel column previously equilibrated with 10 ml homogenization buffer, at 4° C. The column was washed with 10 ml homogenization buffer and then the bound enzyme was eluted with 3 ml of homogenization buffer containing 0.5M NaCl. Total protein concentrations were determined by the method of Bradford (1976).

The PKC activity present in the above-described partially purified cell extracts above was assayed immediately after isolation. The synthetic peptide R-K-R-T-L-R-R-L, corresponding to amino acids 651–658 of the epidermal growth factor receptor (Ullrich et al., 1984), was synthesized on an Applied Biosystems model 430A peptide synthesizer, purified by high-performance liquid chromatography, lyophilized, and stored at −20° C. The Threonine at position 654 is an in vivo substrate for PKC (Hunter, et al., 1984; Davis and Czech, 1985). This synthetic peptide is a highly specific substrate for PKC activity in vitro. (Watson et al., 1987; Woodgett, et al., 1986).

The purified material was then redissolved in sterile water at a final concentration of 100 uM and used as the phosphoacceptor substrate in the PKC assays. The general method of assay has been published in detail elsewhere (O'Brian et al., 1985). In most cases, 100 uM synthetic peptide was substituted for 2 mg/ml histone III-S as the phosphoacceptor substrate.

Cell extracts were prepared 48 hours after the cells had reached confluence. As shown in Table 1(a), eight of the ten cell lines generated by infection with the pMV7-PKCbetal construct (lines R6-PKC1, R6-PKC3, and R6-PKC5 through R6-PKC10) contained marked increases in PKC activity when compared to the control lines (R6-C1, -C2, and -C3). It is remarkable that cell line R6-PKC3 contained a 53 fold higher level of PKC activity than that present in the control cells. Two of the lines (R6-PKC2 and R6-PKC4), however, did not display a significant increase in PKC activity, yet they presumably had integrated the pMV7-PKC construct as evidenced by their continued G418 resistance. Subsequent studies verified that these two cell lines contained deletions in the cDNA clone encoding PKCbetal.

In additional studies I found that the very high PKC activity in extracts of R6-PKC3 seen in the presence of 1 mM Ca^{2+} and phosphatidylserine was also apparent in the presence of 1mM EGTA, 100 ng/ml TPA and phosphatidylserine. This very high activity was also seen when we employed histone III-S, rather than the above-described synthetic peptide, as the substrate for phosphorylation. Furthermore, even after 24 weeks of continuous growth and serial passage, the cell lines R6-PKC1 through R6-PKC6 displayed essentially the same levels of PKC activity shown in Table 1.

Autophosphorylation and Gel Electrophoresis of PKC

Cell extracts purified as described above were incubated under conditions which favor the autophosphorylation of PKC, as follows. One hundred ug of partially purified protein extract was incubated in a reaction mixture containing 80 ug/ml phosphatidylserine, 1 mM $CaCl_2$ (or 1 mM EGTA and 100 ng/ml TPA), 5 mM $MgCl_2$, and 30 uM ATP containing 100 uCi [gamma-32P]ATP (New England Nuclear, NEG035). The purity of the radioactive ATP is critical to obtain reproducible autophosphorylation of PKC.

Under these conditions, it has been previously shown that PKC undergoes an autophosphorylation reaction which results in the phosphorylation of several sites on the intact enzyme (Walton et al., 1987; Huang et al., 1986, Woodgett and Hunter, 1986; Kikkawa, 1982).

Reactions were incubated at room temperature for 10 minutes and then stopped by the addition of SDS-PAGE loading buffer containing 2-mercaptoethanol. This material was then subjected to discontinuous SDS-PAGE by a modification of the method of Laemmli (1970). Twenty ug of total protein were loaded onto each lane. Following electrophoresis, the gels were fixed in 50% acetic acid, 10% ethanol, dried, and autoradiographed on Kodak XAR-5 film.

Autoradiographs of these gels (FIG. 2) revealed that the extracts prepared from four cell lines that had high PKC activity (R6—PKC1, —PKC3, —PKC5, and —PKC6, see Table 1(a)) displayed a prominent phos-

phorylated protein band which was about 75 kd in size, corresponding to the size of an autophosphorylated preparation of PKC obtained from rat brain (Huang et al., 1986; Housey et al., 1987). When examined in an immunoblot assay this 75 kd band also reacted with an antibody to the betal form of PKC (Jaken and Kiley, 1987). The control cell lines R6—C1, —C2 and —C3, and the cell lines R6—PKC2 and R6—PKC4, which did not have increased levels of PKC (see Table 1(a)) did not show this 75 kd phosphorylated band (FIG. 2), nor did they contain any bands which reacted with the antibody to the betal form of PKC. It is of interest that the samples obtained from the four cell lines that produced high levels of PKC also displayed weaker but distinct phosphorylated protein bands that were about 73, 60, and 49 kd in size, which were not seen (or only faintly detected) in the extracts from cells that did not have increased levels of PKC (FIG. 2). These bands may represent degradation fragments of the 75 kd PKC molecules, or specific cellular proteins that are phosphorylated by PKC.

The above-described phosphorylated protein bands were seen when either 1 mM Ca2+ plus phosphatidylserine or 100 ng/ml TPA plus phosphatidylserine were used as cofactors for PKC activation (compare even and odd numbered lanes in FIG. 2). When, however, extracts from cell lines producing high levels of PKC were incubated in an autophosphorylation reaction in the absence of such oofactors, the 75 kd band and the additional smaller bands described above were not detected. These results, taken together with the negative results obtained with extracts from the control cells (FIG. 2), clearly indicate that the phosphorylated bands reflect PKC activity.

Phorbol Ester (^{3}H-PDBU) Binding Assays

Since it has been shown that PKC is a high affinity intracellular receptor for the phorbol ester tumor promoters (for review, see Nishizuka, 1986), I also assayed a subset of the cell lines for 3H-PDBu binding using a previously described intact cell assay (Horowitz et al., 1981).

Cells were plated at 1×10^5 per 4 cm well on day 1, the medium was changed on day 2 and the cells then assayed on day 3. The monolayer was washed with 4 ml of DMEM (without serum), then 2 ml of DMEM containing 50 nM ^{3}H-PDBU (New England Nuclear; 8.3 Ci/mmol) was added and the plates were incubated for 1 hr at 37° C. to determine total binding. The fraction of the total binding that represented specific binding was determined by the addition of a 1000-fold excess of unlabelled PDBU (LC Services) to the 3H-PDBU stock (Horowitz et al., 1981). The plates were washed 3× with 4 ml ice-cold PBS. The cells were solubilized in 1 ml 1% SDS/10 mM DTT for 2 hrs at 37° C. The lysate was transferred to a scintillation vial and counted. Replica plates were used to determine the number of cells per plate and the specific binding data expressed as nanomoles 3H-PDBu bound/10^6 cells. Scatchard analyses were performed as previously described (Horowitz et al., 1981).

I found that the R6-PKC cell lines 1,3,5 and 6, all of which had high PKC enzyme activity, also had a marked increase in 3H-PDBu binding, whereas the cell line R6-PKC4, which did not display a significant increase in PKC activity, did not show an increase in 3H-PDBu binding when compared to the two control cell lines R6-C1 and R6-C2 (Table 1(a)). Scatchard analyses of the control cell line R6-CI and of the R6-PKC3 cell line, performed as previously described (Horowitz et al., 1981), indicated that the number of high affinity receptors in the two cell lines was 1.6×10^5 and 1.4×10^6, respectively. The affinity constants were approximately the same in both cell lines (Kd = 16 nM). Thus, under the assay conditions used, the R6-PKC3 cells contain about ten times the level of high affinity phorbol-ester binding sites as the control cells. It is apparent that the cell lines that express very high levels of PKC also have a significant increase in phorbol ester binding sites.

Assays for PKC-related RNA transcripts

In view of the above results, it was of interest to analyze the poly A+ RNA fraction of several of the cell lines described in Table 1(a) for the size and abundance of RNA transcripts containing sequences homologous to PKCbetal.

Poly A+ RNA was separated on 1% agarose, 6%-formaldehyde gels, blotted onto nylon membranes, hybridized to a 32P-labelled DNA probe prepared from the full-length PKCbetal cDNA, and autoradiographed, as previously described (Housey, et al., 1986). As shown in FIG. 3, the lines that contained elevated levels of PKC activity (R6—PKC1,—PKC3,—PKC5, and —PKC6, see Table 1(a)) contained a prominent 6.6 kb RNA species which corresponds to the predicted size for a mRNA transcript that initiates in the 5' LTR and terminates in the 3' LTR of the pMV7-PKCbetal construct. This transcript was most abundant in the R6-PKC3 cell line (FIG. 3) which also expresses the highest level of PKC activity (Table 1).

On the other hand, lines R6-PKC2 and R6-PKC4, which showed no significant elevation of PKC activity (Table 1(a)), produced truncated mRNA's of approximately 5 kb and 4.8 kb, respectively. The abundance of the latter transcripts was much lower than that of the 6.6 kb transcripts present in the cell lines that expressed high levels of PKC. The neo+ phenotype and the lack of PKC activity in cell lines R6-PKC2 and R6-PKC4 suggest that the truncated mRNAs reflect deletions in PKC coding sequences in the integrated constructs carried by these cell lines. Indeed, genomic DNA blot hybridizations of clones R6-PKC2 and R6-PKC4 indicated that they contained deletions in the PKCbetal cDNA sequence. Neither in the parental Rat 6 cell line, nor in any of the experimental cell lines derived from these cells, was it possible to detect evidence of an endogenous transcript homologous to the PKCbetal probe (FIG. 3). Thus, in these cells, there is negligible expression of the endogenous gene encoding PKCbetal.

Assays of Growth in Monolayer Culture and in Soft Agar

Cells were seeded at a density of 10^4/plate in a series of 60 mm plates, in 5 ml DMEM plus 10% CS. Twenty-four hours later, cells in triplicate plates were trypsinized and counted. This point was designated "day 0." The remaining cultures were then grown in the respective medium (i.e. plus or minus 100 ng/ml TPA) with fresh medium changes twice per week. Cell counts per plate were then determined on triplicate plates during the remainder of the growth curve (FIG. 3). The results obtained were analyzed for exponential doubling time and saturation density (Table 2). To assess growth in soft agar (anchorage independence), 2×10^4 cells were suspended in 2 ml of 0.3% Bacto agar (Difco Laborato-

ries, Detroit, Mich.) in DMEM containing 20% fetal calf serum (FCS) and overlayed above a layer of 5 ml of 0.5% agar in the same medium, on 60 mm petri dishes. The cells were then overlaid with DMEM plus 20% FCS every 4 days. At the end of 30 days, colonies were stained with the vital stain 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolinium chloride hydrate (INT) (Sigma Chemical Co., St. Louis, Mo.) for 48 hours at 37° C., in an incubator with 5% CO2 (Schaeffer and Friend, 1976), and the number of colonies counted under low power on an inverted phase microscope. The data are expressed as "cloning efficiency", i.e. number of colonies greater than 0.05 mm per plate × 100 divided by the number of cells originally seeded per plate.

Screening of a Known Activator of PKC

We found that cell lines which overproduce PKC display an exaggerated morphologic response to 12-O-tetradecanoyl phorbol-13-acetate (TPA) and altered growth control.

Changes in Morphology

To further characterize the phenotypic changes which occurred in the cell lines that overproduce PKCbetal, lines R6-PKC3 and R6-PKC5, which contain 53- and 20-fold elevations of PKC activity, respectively, were first examined in detail with respect to their morphology, in comparison to the control cell line R6-Cl. As shown in FIG. 4, Panel A, in the absence of TPA treatment all three cell lines showed the characteristic fusiform morphology of monolayer cultures of the normal parental Rat 6 fibroblast cell line. At 24 hours after treatment with 100 ng/ml TPA (FIG. 4, R6-C1: panel D), the control cell line displayed more elongated and dendritic cells and a criss-cross pattern, changes previously seen shortly after rodent fibroblasts are treated with TPA (Boreiko et al., 1980). These changes were, however, much more dramatic when the R6-PKC3 and R6-PKC5 cells were treated with TPA (R6-PKC3 and R6-PKC5, Panel D). This was particularly striking with the R6-PKC3 cells (which express the highest level of PKC) since they displayed very long cytoplasmic processes and numerous refractile cell bodies.

By 48 hours following exposure to TPA the morphology of the control cell line R6-Cl had returned to its normal appearance (FIG. 4, Panel E). On the other hand, the R6-PKC3 cells, and to a lesser extent the R6-PKC5 cells, continued to display an altered morphology. All of the cell lines were then exposed to a second, fresh dose of TPA (100 ng/ml) and examined 24 hours later (FIG. 4, Panel F), i.e., 72 hours after the first dose of TPA. The control cells failed to respond, in terms of morphologic change, to the second dose of TPA whereas the R6-PKC3 cells continued to display their altered morphology as well as an increase in cell density. The R6-PKC5 cells displayed only slight changes in morphology in response to the second dose of TPA.

Presumably, the very high level of constitutive production of PKC in the R6-PKC3 cell line is responsible for their exaggerated morphologic response to TPA as well as the failure of these cells to display the usual refractory response to TPA following an initial exposure. In normal cells, the latter response appears to be due to "down-regulation" of endogenous PKC activity. Since the R6-PKC5 cells have an intermediate level of PKC, it is not surprising that their morphologic responses to TPA are intermediate between those of the control cells and the R6-PKC3 cells.

Growth Curves

It was also of interest to determine the growth rates of these cells in monolayer culture. Detailed growth curves were performed on R6-Cl, R6-PKC3 and R6-PKC5 cells in 10% calf serum and DMEM medium, in the absence and presence of 100 ng/ml TPA. The data obtained are summarized quantitatively in Table 2.

In the absence of TPA, the R6-Cl control cell line displayed the longest doubling time (26.4 hours) and the lowest saturation density (3.4×106 cells/plate); the R6-5 cell line had a shorter doubling time (24.9 hours) and a higher saturation density (4.8×10^6 cells/plate); and the R6-PKC3 cell line had the shortest doubling time (24.2 hours) and the highest saturation density (5.7×10^6 cells/plate). The presence of TPA decreased the doubling times, and also increased the saturation densities of all three cell lines, but the enhancement by TPA was particularly striking in the case of R6-PKC3 (Table 2).

When the cell lines were maintained in the presence of TPA for a longer period of time, the R6-PKC3 cells, but not the R6PKC5 or R6-Cl cells, showed a decline in cell density. The latter effect was due to the fact that when the R6-PKC3 cells were maintained at high cell density in the presence of TPA they became less adhesive and tended to detach from the plate.

Thus, even in the absence of TPA the R6-PKC3 cells, (which have the highest level of PKC), and to a lesser extent the R6-PKC5 cells (which have an intermediate level of PKC), exhibit an enhancement of their growth properties which is even greater than that seen when the R6-Cl control cells are grown in the presence of TPA. Moreover, in the presence of TPA these differences in growth properties between the control and R6-PKC3 cells are even more striking.

Foci Formation

In additional studies, monolayer cultures were maintained at post-confluence for an extended period of time (28 days), with media changes every 3 days, in the absence of TPA. Whereas the control R6-C2 cell line remained a fairly uniform monolayer, after about 21 days the R6-PKC3 cell line developed numerous dense foci which were approximately 0.1–0.3 mm in diameter (FIG. 5). Furthermore, the R6-PKC3 cultures displayed numerous cells with a highly vacuolated cytoplasm which were scattered throughout the monolayer, but were not seen in the R6-C2 control culture. When the dense foci seen in the R6-PKC3 culture were picked and further passaged they grew like the parental R6-PKC3 cells and did not display a morphology typical of malignantly transformed cells. It may be surmised that these dense foci, and the vacuolated cells, reflect physiologic rather than genetic changes induced by the high level of PKC activity.

Growth on Soft Agar

I also assayed these cell lines for their ability to form colonies in soft agar, since with rodent cells the acquisition of anchorage-independent growth often correlates with tumorigenicity (Freedman and Shin, 1974). As shown in FIG. 6, when 2×10^4 cells were plated in 0.3% soft agar, both the R6-PKC3 and the R6-PKC5 cells formed numerous small colonies, whereas the control R6-Cl cells (and the parental Rat 6 cell line) failed to

grow and persisted as single cells. In addition, when TPA (100 ng/ml) was added to the agar medium, the colony sizes and cloning efficiencies of the R6-PKC3 and RC-PKC5 cells were enhanced (FIG. 6 and Table 2), but the R6-CI cells still failed to grow in agar.

The cloning efficiencies and colony sizes of the R6-PKC3 cultures were always greater than those of the R6-PKC5 cultures, both in the absence and presence of TPA (Table 2), presumably reflecting the higher level of PKC activity in the former cell line. Thus, it is clear that the overproduction of PKC is associated with the acquisition of anchorage-independent growth in Rat 6 cells. The sizes of the colonies formed in agar by the PKC cell lines are smaller than those formed by Rat 6 cells transformed by an activated c-H-ras oncogene, which have a diameter of about 0.5–1.0 mm.

Further Screening of Activators and Inhibitors of PKC

Additional compounds tested included the tumor promoters teleocidin, aplysiatoxin, and mezerein, all of which are known activators of PKC (O'Brian, et al., CSH, 1985). Furthermore, a known inhibitor of PKC, H-7 (Kawamoto and Hidaka, 1984), also modulated the growth of the cells in the expected manner.

Moreover, this method has been used to establish that the anti-estrogen tamoxifen (O'Brian, et al, Cancer Res., 1985), which inhibits PKC enzyme activity in a cell-free assay, is capable of completely inhibiting the growth in agar of all of the cell lines overproducing PKC. Inhibition of the growth of the R6-PKC3 cells in agar in the presence of tamoxifen provided critical evidence that tamoxifen could inhibit PKC-mediated stimulation of cellular growth.

Furthermore, the concentration of the inhibitor necessary to completely inhibit the growth of each cell line was roughly proportional to the amount of PKC being overproduced in that particular cell line. In other words, there is a direct relationship between the molar amount of inhibitor required to prevent cell growth and the molar amount of PKC present in each cell line.

In addition, I have demonstrated the analogous relationship between the molar amounts of PKC activators and the molar amounts of PKC present in cells. In other words, there is a direct relationship between the molar amount of activator required to stimulate cell growth and the molar amount of PKC present in cells. Thus, this work establishes, for the first time, the fact that stable overproduction of a protein in mammalian cells can result in a novel cellular phenotype(s) (in this case anchorage independence) which can be directly modulated by chemical agents which interact with the protein.

EXAMPLE 2

If one were interested in screening for a potent inhibitor of the c-H-ras oncogene product (the p21 protein) then one would generate cells which grow well in soft agar with appropriate media conditions when p21 is stably overproduced at a certain level, but not at all when p21 is present at wild type levels. Screening for a potent p21 inhibitor could then be performed as described in Example 1 above.

EXAMPLE 3

The same basic techniques would also apply to genes (or cDNA sequences) which have been mutated either by laboratory design (e.g. site-directed mutagenesis) or as a result of naturally occurring events. Thus, any of the known point mutations in the ras oncogene which result in greater capability by the mutated gene to transform normal cells to cancerous ones could be employed in the same basic procedures as described above.

EXAMPLE 4

The previously mentioned retroviral expression vector pMV7 was utilized to introduce a cDNA encoding the betaI isoform of PKC into FDC-P1 cells. FDC-P1 is a bone marrow-derived cell line which requires interleukin-3 (IL-3) for growth. In these studies, the hemopoietic cell line FDC-P1 was utilized in order to demonstrate the utility of the screening method even in cells which have very stringent growth requirements and would otherwise be very difficult to maintain in culture.

The following abbreviations are used herein: SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis (beta aminoethyl ether) N,N,N',N'-tetraacetic acid; EDTA, ethylene diamine tetraacetic acid; TPA, 12-O-tetradecanoyl phorbol-13-acetate (also known as PMA); CHO, Chinese hamster ovary cells; DEAE, diethyl aminoethyl.

Cell Culture

FDC-P1 cells were routinely maintained in Dulbecco's modified eagle's medium containing 10% iron-supplemented calf serum. We added 10% WEHI-3B cell-contained medium as a source of IL-3 and subcultured the cells twice weekly to give 2×10E5 cells/ml.

Isolation of cell lines stably overexpressing PKC

Subconfluent [PSI]-2 cells expressing a cDNA clone encoding the complete PKCbetaI sequence (Housey et al., 1988) and containing the G418 resistance gene were irradiated with 3000 rad. These cells were cocultivated with FDC-P1 grown as described above, and 8 ug/ml of polybrene was added. After 48 hours, the FDC-P1 cells were pelleted, plated in 35mm petri dishes containing 0.3% agar, 10% calf serum, and 10% WEHI conditioned medium, and selected with 0.3 mg/ml of G418. After 10 days colonies were aspirated from the agar and grown in liquid medium.

RNA isolation and blot hybridization

Total RNA was extracted (Chomczynski, 1987) from FDC-P1 cells and run on a 1.5% agarose gel, blotted to nitrocellulose, and probed with the nick-translated EcoRI-PstI fragment of the RP-58 cDNA encoding PKCbetaI (Housey, 1988). In separate experiments nitrocellulose blots were probed with either the BamHI fragment of the murine GM-CSF cDNA (Lee, 1985) or the XhoI fragment of the murine IL-3 gene (Yokota, 1984), clones donated by Dr. Lee and labelled by nick-translation to 1×10E8 cpm/ug. The labelled PstI fragment of tubulin was used to examine RNA loading.

Western blotting of PKC

FDC-P1 cells were placed directly into a buffer containing 1% SDS; 2 mM EGTA; 2mM EDTA; 20 mM Tris-HCl, pH 8.0, and 1% beta mercaptoethanol. The sample was boiled and pelleted for 30 min. at 20 degrees C at 200,000×g. Then 80 ug of supernatant protein was electrophoresed on a 10% SDS polyacrylamide gel, and the proteins were transferred to nitrocellulose. The blot was blocked with bovine serum albumen and was probed with an antibody made to a conserved region of PKC (LLNQEEGEYYNVPIPE) which recognizes all forms of the enzyme (Stabel, 1987). The PKC standard

was purified from rat brain by previously published procedures (Kraft, 1988).

Stable Overproduction of PKCbetaI in FDC-P1 Cells

In order to generate FDC-P1 cells which stably overproduced PKCbetaI, we cocultured FDC-P1 cells, which are dependent upon a source of IL-3 for continued growth, with [PSI]-2 cells which produce a retrovirus encoding the complete PKC betaI cDNA clone in the pMV7 expression vector. After 48 hours of cocultivation, the FDC-P1 cells were plated in soft agar in medium containing G418 and IL-3, and after 10 days of selection several colonies were aspirated and grown in liquid culture.

Approximately 30 of these clones were screened by western blotting with a PKC antibody which recognizes all forms of PKC. A number of clones demonstrated elevated levels of PKC betaI protein by Western blot analysis. Northern blot analyses were consistent with the western blot results and demonstrated that clones 40, 43, and 51 exhibited hybridizable bands to the EcoRI-PstI fragment of the PKCbetaI clone, whereas the parental cell line and clone 2 (which lacked any PKCbeta 1-specific mRNA), were negative. Furthermore, the total PKC activity in these cells was examined using histone IIIs as a phosphotransferase acceptor (Table 4). As expected, Clone 2 and the parental cell line had approximately the same PKC activity, whereas clones 40, 43, and 51 demonstrated approximately 20-fold increases in activity.

PKC Activators Induce Significant Morphologic Changes in FDC-P1 Cells That Stably Overproduce PKC

We then tested the morphologic responsiveness of the FDC-P1 cells to activators of PKC induce profound morphologic changes in the FDC-P1 cells. Several cell lines were plated at 3×10E4 cells/ml and treated with 100 nM TPA. After 12 hours, representative fields were photographed. The PKC betaI overproducing cells exhibited profound morphologic changes, including a generalized rounding up of the cells followed by the rapid formation of dense, cellular clumping. The latter clumps are easily visualized with standard cell stains such as Giemsa, and stain much darker than the corresponding control cells under these conditions.

Screening Assay to Detect Activators and Inhibitors of PKC

Thus, as with our previous findings for other cell lines such as NIH 3T3, Rat 6, and C3H 10T ½, this system also provides a rapid, powerful cell-based assay for the discovery and characterization of both activators and inhibitors of PKC as follows. Compounds could be rapidly screened for new activators of PKC by testing their ability to induce cellular clumping in the Test cells, while leaving the Control cells essentially unaffected. In addition, if one wanted to screen for PKC inhibitors, compounds could be rapidly screened for their ability to inhibit the effects of PKC by testing their ability to prevent cellular clumping induced by TPA in the Test cells, while again leaving the Control cells essentially unaffected. In this latter test, Control and Test cells would be incubated simultaneously with 100 nM TPA and the individual compounds to be tested. After 12 hours the Control and Test cells would be stained and scored for the inhibition of cellular clumping in the Test cells while leaving the Control cells unaffected.

EXAMPLE 5

In further studies designed to test the utility of this system with other proteins of interest (POI), we have utilized the human insulin receptor as a model POI. Cell lines which stably overproduce the insulin receptor were used to develop an assay system which is capable of detecting both agonists and antagonists of insulin action.

In the majority of vertebrates, insulin is the primary hormone involved in the homeostatic control of blood glucose levels. The many effects of insulin action on cells include stimulation of glucose, protein, and lipid metabolism as well as effects on RNA and DNA synthesis (Metcalf, 1985). These actions of insulin on cells begin at the molecular level through the binding of insulin to the insulin receptor. The insulin receptor is present on virtually all mammalian cells, but its number varies from as few as 40 to more than 200,000 receptors per cell (Kahn and White, 1988, and references therein). The insulin receptor is a heterotetrameric glycoprotein consistent of two alpha and two beta subunits. The alpha subunits, Mr 135,000, are located entirely extracellularly, whereas the beta subunits are transmembrane proteins which possess intrinsic tyrosine kinase activity. The alpha and beta subunits are attached by disulfide bonds on the extracellular side of the cell membrane. Both the alpha and the beta chains of the mature receptor molecule are derived from a single chain precursor polypeptide (the "proreceptor") which in turn is encoded by a single gene.

It is now known that the beta subunit of the insulin receptor is a protein tyrosine kinase which phosphorylates substrate proteins on tyrosine residues. This tyrosine kinase activity of the beta subunit is stimulated by the binding of insulin to the alpha subunit, and it has been shown that the tyrosine kinase activity of the beta subunit is essential for insulin action on target cells (Kahn and White, 1988, and references therein).

Generation of CHO Cell Lines Stably Overproducing the Human Insulin Receptor

In additional demonstrations of the utility of the method, we have employed Chinese Hamster Ovary (CHO) cell lines which stably overproduce the human insulin receptor (HIR) (White et al., 1988). These cell lines were generated using approaches analogous to those previously described for the PKC-overproducing cell lines. However, in these HIR studies we utilized a different expression vector, distinct cell lines, and an alternative method of gene transfer (direct transfection as opposed to virus-mediated transduction as used in the PKC experiments previously described). Briefly, CHO cells were co-transfected with the transfer vector pSVEneo (containing the neomycin resistance gene) and the expression vector pCVSVHIRc as previously described (White et al., 1988). After twenty four hours, the transfected cells were placed into 800 ug/ml G418 to select resistant cells. Approximately two weeks later, G418-resistant clones were isolated and subcloned. Clonal cell lines which express high levels of surface insulin receptors were selected by fluorescence-activated cell sorting. One such line (CHO/HIRC), which stably overproduced approximately 80,000 insulin receptors per CHO cell, was utilized in these experiments. A control CHO cell line (CHO/NEO), harboring only the

pSVEneo transfer vector, were generated in analogous fashion. Detailed preliminary characterizations of this cell line have been described (White et al., 1988).

Morphologic and Growth Control Effects of Insulin Treatment on CHO Cells Stably Overproducing the Human Insulin Receptor

We then tested the ability of the CHO/HIRC and CHO/NEO cells to respond to the following insulin treatment protocol. Control cells, hereinafter termed CHO/NEO (which harbor pSVEneo alone), and the Test cells, termed CHO/HIRC (which stably overproduced about 80,000 insulin receptors per cell), were incubated in the presence and absence of insulin. Control (CHO/NEO) and Test (CHO/HIRC) cell lines were exposed to 400 nM of insulin and the resultant morphologic changes were observed and photographed using a Bausch and Lomb BL-200 phase-contrast microscope with a Nikon FM-200 camera attached.

After 18 hours the test cells began to exhibit profound morphologic changes, including increased refractility, cellular rounding, and decreased adherence to the culture plate (anchorage dependence). These effects were further pronounced at 24 hours after exposure. Indeed, by 48 hours after treatment, virtually all of the test cells had completely detached from the plate, whereas the control cells were still largely intact. If the test cells were transferred to new plates in medium lacking high levels of insulin, however, they were completely viable and would attach to the plate and continue to grow normally. Thus, although insulin treatment could rapidly induce the anchorage independent phenotype, the cells remained completely viable for future growth if insulin was subsequently removed from their growth medium.

Screening Assay to Detect Activators and Inhibitors of the Insulin Receptor

Thus, in yet another demonstration of the utility of our method, the system described above provides a rapid and powerful screening method for the discovery and characterization of insulin agonists and antagonists as follows.

Compounds may be screened by testing their ability to preferentially induce anchorage-independent growth in the Test cells relative to the Control cells. Each compound would be applied to both the Control and the Test cells growing in multi-well plates, and 48 hours later the wells would be stained with a simple dye such as Giemsa (Housey et al., 1988 and references therein).

Whereas the Control CHO/NEO cells would consistently remain attached to the plate and stain strongly with Giemsa, the Test CHO/HIRC cells would be induced to detach from the plate by compounds which mimic the effects of insulin and therefore would no longer be stained by Giemsa. A standard microtitre well plate-reader (such as those produced by SLT America, Inc.) could easily read the absorbance of each well due to the staining procedure(s) used.

Thus, in this manner thousands of compounds could be screened for their ability to mimic the effects of insulin. Such agents are, of course, insulin agonists. However, as with the methods described above for PKC, the method could also be utilized for the discovery of insulin antagonists by simply treating the Control and Test Cells with potential antagonist compounds in the presence of insulin.

By this latter approach, compounds capable of inhibiting the effects of insulin action would prevent the induction of anchorage independence in the CHO/-HIRC cells, whereas compounds with no effect would be unable to prevent the induction of anchorage-independent growth in the Test Cells. Most importantly, this insulin receptor assay system demonstrates yet another direct application of the utility of stable overproduction of a protein-of-interest (POI) for the development of powerful assay systems capable of detecting activators or inhibitors of any POI.

Julius, et al., Science, 244: 1057–62 (Jun. 2, 1989) reports the production of a cell line which overproduces the serotonin receptor 5HTlc by cloning its cDNA into the expression vector pMV7. The transformed cell lines exhibited a change in morphology, specifically, the formation of foci. Mesulergine, a serotonin antagonist, completely blocked the formation of foci. This paper was published after the filing date of the parent application, Feb. 10, 1988, and is therefore cited merely to further demonstrate the broad applicability of the screening method described herein.

Further Modifications

Use of any expression vector capable of stably overexpressing a given gene in a recipient cell could be used with success in the procedures described herein. The retroviral vector which I used here was particularly well suited to the problem since I had designed it specifically for these purposes. However, other similar vector systems would work. Also, one could do co-transfection of an experimental gene inserted in one plasmid vector along with a second plasmid containing the selectable marker gene (rather than having both the experimental gene and the selectable marker gene on the same plasmid vector). This is more difficult and less efficient than using the pMV7 vector, but it would work to some extent.

Any growth medium, in addition to soft agar or methocel, which tends to prohibit the growth of normal, non-transformed cells, could also be used.

A culture of *E. coli* DH1 bearing the plasmid denoted pMV7-RP58 (pMV7-PKC beta1), was deposited under the Budapest Treaty with the American Type Culture Collection on Feb. 11, 1988, ATCC No. 67654. The deposit of this plasmid is not to be construed as an admission that the deposit is required for enablement or that the disclosure is limited to the deposited vector or gene.

TABLE 1

PKC Activity and Phorbol Ester Binding in Rat 6 Cells Infected with pMV7 or pMV7-PKCbeta1 Constructs

Cell Line	PKC Activity Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control	^{125}I-PDBU Binding (pmol/10^6 cells)
R6-C1	100	—	1.6
R6-C2	85	—	1.3
R6-C3	120	—	1.5
mean + s.d.	100 ± 34	1	
R6-PKC1	2480	23	12.7
R6-PKC2	85	1	ND
R6-PKC3	5840	53	9.9
R6-PKC4	190	2	0.7
R6-PKC5	2200	20	5.8
R6-PKC6	4400	42	7.1
R6-PKC7	2150	20	ND
R6-PKC8	3280	30	ND
R6-PKC9	4890	45	ND

TABLE 1-continued

R6-PKC10	5050	46	ND

PKC Activity in NIH-3T3 Cell Lines Infected with pMV7 or pMV7-PKCbetaI Constructs

NIH-3T3 Cell lines (Controls)	PKC Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control
3T3-C1	110	—
3T3-C2	150	—
3T3-C3	90	—
mean +/− s.d. (control lines)	115 +/− 30	
NIH-3T3 PKC-Overprodecing Cell Lines		
3T3-PKC1	2570	22
3T3-PKC2	3640	32
3T3-PKC3	1960	17
3T3-PKC4	1240	11
3T3-PKC5	4190	36
3T3-PKC6	2110	18
3T3-PKC7	5050	44

PKC Activity in C3H-10T1/2 Cell Lines Infected with pMV7 or pMV7-PKCbetaI Constructs

NIH-3T3 Cell lines (Controls)	PKC Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control
C3H-C1	115	—
C3H-C2	155	—
C3H-C3	130	—
C3H-C4	185	—
mean +/− s.d.	145 +/− 30	
C3H-10T1/2 PKC-Overprodecing Cell Lines		
C3H-PKC1	2340	16
C3H-PKC2	5010	35
C3H-PKC3	950	7
C3H-PKC4	1360	9
C3H-PKC5	4340	30
C3H-PKC6	7460	51

Legend to Table 1

Control cell lines were obtained by infecting rat-6-fibroblast, NIH-3T3 and C3H-10T1/2 cells with the pMV7 vector itself (lacking the PKC cDNA insert) whereas the PKC-overprodecing cell lines were obtained from rat-6-fibroblast, NIH-3T3 and C3H-10T1/2 cells infected with the pMV7-PKCbeta construct, as described above. Total PKC activity was partially purified from each of the cell lines and assayed in the presence of 1 mM $Ca2+$ and 80 ug/ml phosphatidylserine, using the synthetic peptide R-K-R-T-L-R-R-L as substrate. Specific activity is reported as the amount of incorporation of $32P$ into the synthetic peptide substrate per milligram of protein per minute. All assays were done in duplicate and varied by less than 10%.

TABLE 2

GROWTH PROPERTIES OF RAT 6 CELL LINES OVERPRODUCING PKC AND THEIR RESPONSES TO TPA TREATMENT

		Growth in Culture			
		Monolayer		Agar	
Cell Line	TPA Add	Doubling Time (hrs)	Saturat. Density ($\times 10^6$)	Effic. (%)	Colony Size (mm)
R6C1	−	26.4	3.4	0	—
(control)	+	24.6	4.2	0	—
R6-PKC3	−	24.2	5.7	25.1	0.10–0.15
(test)	+	21.5	10.0	29.7	0.15–0.35
R6-PKC5	−	24.9	4.8	17.3	0.05–0.10
(test)	+	22.9	7.0	34.7	0.10–0.15

Table 2.

The cells were grown as described above. The "doubling times" relate to the initial exponential phase of cell growth and the "saturation density" represents the number of cells per 6 cm plate on day 11. The data are taken from the experiment shown in FIG. 3.

TABLE 3

Inhibition of Growth in Agar of PKC-Overproducing Cell Lines Using Various Inhibitors

Inhibitor Agar (%)	Cell Line	Conc. Inhibitor (uM)	Effic. of Growth In
H-7	R6-PKC3	0	27
H-7	R6-PKC3	2	25
H-7	R6-PKC3	5	22
H-7	R6-PKC3	10	17
H-7	R6-PKC3	50	4
H-7	R6-PKC3	100	0
H-7	R6-PKC5	0	20
H-7	R6-PKC5	2	21
H-7	R6-PKC5	5	16
H-7	R6-PKC5	10	4
H-7	R6-PKC5	50	1
H-7	R6-PKC5	100	0
Tamoxifen	R6-PKC3	0	25
Tamoxifen	R6-PKC3	5	27
Tamoxifen	R6-PKC3	20	22
Tamoxifen	R6-PKC3	50	15
Tamoxifen	R6-PKC3	100	6
Tamoxifen	R6-PKC3	200	0
Tamoxifen	R6-PKC5	0	19
Tamoxifen	R6-PKC5	5	19
Tamoxifen	R6-PKC5	20	17
Tamoxifen	R6-PKC5	50	8
Tamoxifen	R6-PKC5	100	0
Tamoxifen	R6-PKC5	200	0
Staurosporine	R6-PKC3	0	29
Staurosporine	R6-PKC3	0.001	29
Staurosporine	R6-PKC3	0.005	26
Staurosporine	R6-PKC3	0.010	15
Staurosporine	R6-PKC3	0.050	7
Staurosporine	R6-PKC3	0.250	0
Staurosporine	R6-PKC5	0	22
Staurosporine	R6-PKC5	0.001	21
Staurosporine	R6-PKC5	0.005	17
Staurosporine	R6-PKC5	0.010	10
Staurosporine	R6-PKC5	0.050	2
Staurosporine	R6-PKC5	0.250	0

TABLE 4

PKC Activity in FDC-P1 Cells Transfected with a Retrovirus Encoding PKCbetaI

Cell Line	PKC Activity (pmol/min/ug prot)	Fold Increase Relative to Parental Cell Line
Parental Control	78	1
FDC-P1 clone 2	64	1
FDC-P1 clone 4	1873	24
FDC-P1 clone 43	1558	20
FDC-P1 clone 51	1135	15

Approximately 3 x $10E7$ cells in the logarithmic phase of growth were homogenized, supernatants ghom over a DEAE-cellulose, and eluates assayed as previously described (Kraft, 1983).

REFERENCES

Ashendel, C. The Phorbol Ester Receptor: a phospholipid-regulated protein kinase. (1984) *Biochim. Biophys. Acta* 822, 219–242.

Bollag, G. E., Roth, R. A., Beaudoin, J., Mochly-Rosen, D., Koshland, D. E. Jr. (1986) Protein kinase C directly phosphorylates the insulin receptor in vitro and reduces its protein-tyrosine kinase activity. *Proc. Natl. Acad Sci. USA* 83, pp 5822–4

Boreiko, C.; Mondal, S., Narayan, S., and Heidelberger, C. (1980) Effect of 12-O-Tetradecanoylphorbol-13-acetate on the Morphology and Growth of C3H/10T1/2 Mouse Embryo Cells. Cancer Res. 40, 4709–4716.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein

utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.

Brandt, S. J., Niedel, J. E., Bell, R. M., Young, W. S. (1987) Distinct patterns of expression of different protein kinase C mRNAs in rat tissues. *Cell* 49, pp 57–63.

Catino, J. J., Francher, D. M., Edinger, K. J., and Stringfellow, D. A. (1985) A microtitre cytotoxicity assay useful for the discovery of fermentation-derived antitumor agents. *Cancer Chemother. Pharmacol.* 15, 240–243

Chomczynski, P., Sacchi, N. (1987), *Anal. Biochem.*, 162: 156–159.

Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U., & Ullrich, A. (1986) Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signalling pathways. *Science* 233, 859–866.

Dailey, L., and Basilico, C. (1985). Sequences in the polyomavirus DNA regulatory region involved in viral DNA replication and early gene expression. *J. Virol.* 54, 739–749.

Daley, G. Q., McLaughlin, J., Witte, O. N., Baltimore, D. The CML-Specific P210 bcr/abl Protein, Unlike v-abl, Does Not Transform NIH/3T3 *Fibroblasts.Science* 237, pp 532–535.

Davis, R. J., and Czech, M. P. (1985) Platelet-derived growth factor mimics phorbol diester action on epidermal growth factor receptor phosphorylation at threonine 654. *Proc. Natl. Acad. Sci.* 82, 4080–4084.

Freeman, A. E., Price, P. J., Igel, H. J., Young, J. C., Maryak, J. M. Huebner, R. J. (1970) Morphological transformation of rat embryo cells induced by dimethylnitrosamine and murine leukemia viruses. *J. Natl. Cancer Inst.* 44, pp 65–78.

Freedman, V. H. and Shin, S. (1974) Cellular Tumorigenicity in nude Mice: Correlation with Cell Growth in Semi-Solid Medium. *Cell* 3, 355–359.

Graham, F. L., and van der Eb, A. J. (1973) A new technique for the assay of infectivity of human adenovirus DNA. *Virology* 52, 456–467.

Gould, K. L., Woodgett, J. R., Cooper, J. A., Buss, J. E., Shalloway, D., Hunter, T. (1985) Protein Kinase C Phosphorylates pp60src at a novel site. *Cell* 42, pp 849–857.

Horowitz, A. D., Greenebaum, E. and Weinstein, I. B. (1981) Identification of receptors for phorbol ester tumor promoters in intact mammalian cells and of an inhibitor of receptor binding in biologic fluids. *Proc. Natl. Acad. Sci. USA* 78, pp 2315–2319

Housey. G. M., O,Brian, C. A., Johnson, M. D., Kirschmeier, P., and Weinstein, I. B. (1987) Isolation of cDNA clones encoding protein kinase C: Evidence for a protein kinase C-related gene family. *Proc. Natl. Acad. Sci. USA* 84, pp 1065–1069

Housey, G. M., Kirschmeier, P., Garte, S. J., Burns, F., Troll, W., & Weinstein, I. B. (1985) Expression of long terminal repeat (LTR) sequences in carcinogen-induced murine skin carcinomas. *Biochem. Biophys. Res. Commun.* 127, 391–398.

Hsiao, W.-L. W., T. Wu, Weinstein, I. B., (1986) Oncogene-Induced Transformation of a Rat Embryo Fibroblast Cell Line is Enhanced by Tumor Promoters. *Mol. Cell. Biol.* 6, pp 1943–1950

Hunter, T., Ling, N., Cooper, J. A. (1984) Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane *Nature* 311, 480–3

Huang, K. P., Nakabayashi, H., Huang, F. L. (1986) Isozymic forms of rat brain Ca2+-activated, phospholipid-dependent protein kinase. *Proc. Natl. Acad. Sci.* 83, 8535–8539.

Housey, G. M., Johnson, M. D., Hsiao, W. L., O'-Brian, C. A., Murphy, J. P., Kirschmeier, P. and Weinstein, I. B., (1988), *Cell*, 52: 343–54.

Jaken, S. and Kiley, S. (1987) Purification and characterization of three types of protein kinase C from rabbit brain cytosol. *Proc. Natl. Acad. Sci USA* 84, pp 4418–4422

Jeng, A. Y., Srivastava, S. K., Lacal, J. C., Blumberg, P. M. (1987) Phosphorylation of ras oncogene product by protein kinase C. Biochem. Biophys. Res. Commun. 145, pp 782–8.

Johnson, M. D., Housey, G. M., Kirschmeier, P., and Weinstein, I. B. (1987) Molecular Cloning of Gene Sequences Regulated by Tumor Promoters Through Protein Kinase C. *Mol. Cell Biol.* 7, 2821–2829

Kahn, C.R., White, M.F. (1988), The Insulin Receptor and the Molecular Mechanism of Insulin Action, *J. Clin. Invest.*, 82: 1151–56.

Kajikawa, N., Kishimoto, A., Shiota, M., & Nishizuka, Y. (1983) Ca2+-dependent neutral protease and proteolytic activation of Ca2+-activated, phospholipid-dependent protein kinase. *Methods. Enzymol.* 102, 279–290.

Kawamoto, S. and Hidaka, H. (1984) 1-(5-Isoquinolinesulfonyl) -2-methyl-piperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. *Biochem. Biophys. Res. Commun.* 125, 258.

Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982) Calcium-activated, phospholipid-dependent protein kinase from rat brain. Subcellular distribution, purification, and properties. *J. Biol. Chem.* 257, 13341–13348.

Kirschmeier, P. T., Housey, G. M., Johnson, M. D., Perkins, A.S., and Weinstein, I. B. (1988) Construction and Characterization of a Retroviral Vector Demonstrating Efficient Expression of Cloned cDNA Sequences. DNA, in press.

Knopf, J. L., Lee, M-H, Sultzman, L. A., Kriz, R. W., Loomis, C. R., Hewick, R. M. & Bell, R. (1986) Cloning and expression of multiple protein kinase C cDNAs. *Cell* 46, 35 491–502.

Kraft, A. S., Reeves, J. A. and Ashendel, C. L. (1988), *J. Biol. Chem.*, 263: 8437–42.

Kraft and Anderson, W. B. (1983), *J. Biol. Chem.*, 258: 9178–9183.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

Leach, K. L., James, M. L., & Blumberg, P. M., (1983) Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol. *Proc. Natl. Acad. Sci. USA* 80, 4208–4212.

Lee, F., Yokota, T., Otsuka, T., Gemmell, L., Larson, N., Luh, J., Arai, K-I., and Rennick, D. (1985), *Proc. Nat. Acad. Sci. USA*, 82: 4360–64.

Lusky, M., and Botchan, M. (1981). Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. *Nature* 293, 74–81.

Maddon, P. J., Dalgleish, A. G., McDougal, J. S., Clapham, P. R., Weiss, R. A., Axel, R. (1986) The T4 Gene Encodes the AIDS Virus Receptor and Is Expressed in the Immune System and the Brain. *Cell* 47, pp 333–348

Makowske, M., Birnbaum, M. J., Ballester, R., Rosen, O. M. (1986) A cDNA encoding PKC identifies two species of mRNA in brain and GH3 cells. *J. Biol. Chem.* 261, pp 13389–13392

Maniatis, T., Fritsch, E. F., & Sambrook, J. eds. (1983) in Molecular Cloning : A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.)

Mann, R., Mulligan, R. C., and Baltimore, D. (1983) Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* 33, 153–159

Nishizuka, Y. (1986) Studies and Perspectives of Protein Kinase C. Science 233, 305–312.Nishizuka, Y. (1984) The Role of Protein Kinase C in Cell Surface Transduction and Tumour Promotion. *Nature* (London) 308, 693–698.

Metcalf. D., (1985) *Science*. 229, 16–22.

O'Brian, C. A., Lawrence, D. S., Kaiser, E. T., & Weinstein, I. B. (1984) Protein kinase C phosphorylates the synthetic peptide Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val in the presence of phospholipid plus either Ca2+ or a phorbol ester tumor promoter. *Biochem. Biophys. Res. Commun.* 124, 296–302.

O'Brian, C., Arcoleo, J., Housey, G. M., & Weinstein, I. B. (1985) in Cancer Cells 3, eds. Feramisco, J., Ozanne, B. & Stiles, C. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) 359–363.

O'Brian, C. A., Liskamp, R. M., Solomon, D. H. and Weinstein, I. B. (1985) Inhibition of Protein Kinase C by Tamoxifen. *Cancer Res.* 45, 2462–2465

Ono, Y., Kurokawa, T., Fujii, T., Kawahara, K., Igarashi, K., Kikkawa, U., Ogita, K., Nishizuka, Y. (1986) Two types of complementary DNAs of rat brain protein kinase C. FEBS 206, 347–52

Ono, Y., Kikkawa, U., Ogita, K., Tomoko, F., Kurokawa, T., Asaoka, Y, Sekiguchi, K., Ase, K., Igarashi, K., Nishizuka, Y. (1987) Expression and Properties of Two Types of Protein Kinase C: Alternative Splicing from a Single Gene. *Science* 236, pp 1116–1120.

Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T., Hidaka, H. (1987) Tissue-specific expression of three distinct types of rabbit protein kinase C. *Nature* (London) 325, pp 161–6.

Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., & Ullrich, A. (1986) The complete primary structure of protein kinase C—the major phorbol ester receptor. *Science* 233, 853–858.

Perkins, A. S., Kirschmeier, P. T., Gattoni-Celli, S., and Weinstein, I. B. (1983) . Design of a retrovirus-derived vector for expression and transduction of exogenous genes in mammalian cells. *Mol. Cell. Biol.* 3, 1123–1132.

Pontremoli, S., Melloni, E., Michetti, M., Sparatore, B., Salamino, F., Sacco, O., and Horecker, B. L. (1987) Phosphorylation and proteolytic modification of specific cytoskeletal proteins in human neutrophils stimulated by phorbol-12-myristate 13-acetate. *Proc. Natl. Acad. Sci.* 84, 3604–3608.

Sibley, D. R., Benovic, J. L., Caron, M. G., Lefkowitz, R. J. (1987) Regulation of transmembrane signalling by receptor phosphorylation. *Cell* 48, 913–922.

Stabel, S., Rodriguez-Pena, A., Young S., Rozengurt E., and Parker, pJ. (1987), J. Cell Physiol., 130: 111–117.

Uehara, Y., Hori, M., Takeuchi, T., Umezawa, H. (1985) Screening of Agents Which Convert 'Transformed Morphology' of Rous Sarcoma Virus-Infected Rat Kidney Cells to 'Normal Morphology': Identification of an Active Agent as Herbimycin and its Inhibition of Intracellular src Kinase. *Jpn. J. Cancer Res.* 76, 672–675.

Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., and Seeburg, P. H. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309, 418–425.

Walton, G. M., Bertics, P. J., Hudson, L. G., Vedvick, T. S., Gill, G. N. (1987) A Three-Step Purification Procedure for Protein Kinase C: Characterization of the Purified Enzyme. Anal. *Biochem.* 161, 425–437.

Weinstein, I. B., (1987) Growth Factors, Oncogenes, and Multistage Carcinogenesis. *J. Cell. Biochem.* 33, pp 213–224.

White, M. F., Livingston, J. N., Backer, J. M., Lauris, V., Dull, T. J., Ullrich, A., Kahn, C. R., Mutation of the Insulin Receptor at Tyrosine 960 Inhibits Signal Transmission but Does Not Affect its Tyrosine Kinase Activity (1988), *Cell.* 54: 641–649.

Wigler, M., Silverstien, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C, and Axel, R. (1977). Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* 11, 223–232.

Woodgett, J. R., Gould, K. L., and Hunter, T. (1986) Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. *Eur. J. Biochem.* 161 177–184.

Yokota, T., Lee, F., Rennick, D., Hall, C., Aria, N., Mosmann, T., Nabel, G., Cantor, H., and Arai, K-I., (1984), *Proc. Nat. Acad. Sci. USA* 81: 1070–74.

Young, S., Parker, P. J., Ullrich, A., and Stabel, S. (1987) Down-regulation of protein kinase C is due to an increased rate of degradation. *Biochem. J.* 244, 775–779.

Von Hoff, D.D., Forseth, B., and Warfel, L.E. (1985) Use of a Radiometric System to Screen for Antineoplastic Agents: Correlation with a Human Tumor Cloning System. *Cancer Res.* 45, 4032–4036.

I claim:

1. A test kit for determining whether a substance is an inhibitor or activator of a protein whose production evokes a responsive change in a phenotypic characteristic other than level of said protein in said cell per se, which comprises:

(a) a first mammalian cell line which produces the protein and exhibits said phenotypic response thereto; and

(b) a second mammalian cell line which produces the protein at a lower level than the first cell line, or does not produce the protein, and which exhibits said phenotypic response to the protein to a lesser degree or not at all.

2. A test kit for determining whether a substance is an inhibitor or activator of a protein whose production evokes a responsive change in a phenotypic characteristic other than level of said protein in said cell per se, which comprises:

(a) a first cell line which produces the protein and exhibits said phenotypic response thereto;

(b) a second cell line which produces the protein at a lower level than the first cell line, or does not pro-

duce the protein, and which exhibits said phenotypic response to the protein to a lesser degree or not at all; and

(c) a reference reagent comprising a known inhibitor or activator of the protein.

3. A method of determining whether a substance is an inhibitor or activator of a protein whose production by a cell evokes a responsive change in a phenotypic characteristic other than the level of said protein in said cell per se, which comprises:

(a) providing a first FDC-P1 cell line which produces the protein and exhibits said phenotypic response thereto;

(b) providing a second FDC-P1 cell line which produces the protein at a lower level than the first cell line, or does not produce the protein at all, and which exhibits said phenotypic response to the protein to a lesser degree or not at all;

(c) incubating the substance with the first and second FDC-P1 cell lines; and

(d) comparing the phenotypic response of the first FDC-P1 cell line to the substance with the phenotypic response of the second FDC-P1 cell line to the substance.

4. The method of claim 3 wherein said first cell line is obtained by introducing a gene encoding the protein of interest into a FDC-P1 host cell, said gene being under the control of a promoter functional in the FDC-P1 cell, whereby said gene is expressed.

5. A method of determining whether a substance is an inhibitor or activator of a protein whose production by a cell evokes a responsive change in a phenotypic characteristic other than the level of said protein in said cell per se, which comprises:

(a) providing a first CHO cell line which produces the protein and exhibits said phenotypic response thereto;

(b) providing a second CHO cell line which produces the protein at a lower level than the first cell line, or does not produce the protein at all, and which exhibits said phenotypic response to the protein to a lesser degree or not at all;

(c) incubating the substance with the first and second CHO cell lines; and

(d) comparing the phenotypic response of the first CHO cell line to the substance with the phenotypic response of the second CHO cell line to the substance.

6. The method of claim 5 wherein said first cell line is obtained by introducing a gene encoding the protein of interest into a CHO host cell, said gene being under the control of a promoter functional in the CHO cell, wherein said gene is expressed.

7. A method of determining whether a substance is an inhibitor or activator of an insulin receptor which comprises:

(a) providing a first cell line which produces an insulin receptor and exhibits a phenotypic response thereof;

(b) providing a second cell line which produces the insulin receptor at a lower level than the first cell line, or does not produce the insulin receptor at all, and which exhibits a phenotypic response to the insulin receptor to a lesser degree or not at all;

(c) incubating the substance with the first and second cell lines; and

(d) comparing the phenotypic response of the first cell line to the substance with the phenotypic response of the second cell line to the substance.

8. A method of determining whether a substance is inhibitor or activator of a serotonin receptor, which comprises:

(a) providing a first cell line which produces a serotonin receptor and exhibits a phenotypic response thereto;

(b) providing a second cell line which produces the serotonin receptor at a lower level than the first cell line, or does not produce the serotonin receptor at all, and which exhibits a phenotypic response to the serotonin receptor to a lesser degree or not at all;

(c) incubating the substance with the first and second cell lines; and

(d) comparing the phenotypic response of the first cell line to the substance with the phenotypic response of the second cell line to the substance.

* * * * *

US005688655A

United States Patent [19]

Housey

[11] Patent Number: 5,688,655

[45] Date of Patent: *Nov. 18, 1997

[54] METHOD OF SCREENING FOR PROTEIN INHIBITORS AND ACTIVATORS

[75] Inventor: Gerard Housey, New York, N.Y.

[73] Assignee: ICT Pharmaceuticals, Inc., New York, N.Y.

[*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. No. 4,980,281.

[21] Appl. No.: 408,443

[22] Filed: Mar. 17, 1995

Related U.S. Application Data

[63] Continuation of Ser. No. 977,986, Nov. 18, 1992, abandoned, which is a continuation of Ser. No. 392,073, Aug. 10, 1989, Pat. No. 5,266,464, which is a continuation-in-part of Ser. No. 154,206, Feb. 10, 1988, Pat. No. 4,980,281.

[51] Int. Cl.[6] C12Q 1/02

[52] U.S. Cl. 435/7.21; 435/29; 435/172.3

[58] Field of Search 435/7.4, 29, 172.3

[56] References Cited

U.S. PATENT DOCUMENTS

4,480,038	10/1984	Cheng .	
4,532,204	7/1985	Crespi .	
4,569,916	2/1986	Penman et al.	435/29
4,701,406	10/1987	Chou	435/7
4,859,585	8/1989	Sonnenschein et al.	435/29
4,910,132	3/1990	Knight et al.	435/29
4,980,281	12/1990	Housey	435/240.1
5,242,185	9/1993	Lam et al.	435/6
5,266,464	11/1993	Housey	435/29

FOREIGN PATENT DOCUMENTS

WO-A-8 907 654	8/1989	WIPO .

OTHER PUBLICATIONS

Gerard M. Housey, et al., 'Overproduction of Protein Kinase C Causes Disordered Growth Control In Rat Fibroblasts', *Cell*, vol. 52, 12 Feb. 1988, Cambridge, NA US pp. 343–354.

Julius, et al., Ectopic Expression of The Serotonin 1c Receptor and the Triggering of Malignant Transformation (1989) *Science*, vol. 244, pp. 1057–1062.

R.S. Krauss, et al ., Disturbances in Growth Control and Gene Expression in a C3H/10T 1/2 Cell Line that Stably Overproduces Protein Kinase C, *Oncogene*, vol. 4, 1989. pp. 991–998.

Enhancement of Extracellular Enzyme Secretion in Bacillus Licheniformis NCIB 6346 Author: Ailsa P. H. Grieveson, 1987.

Armelin et al., (Nature 310, 1984, pp. 655–660).

Huang et al. (Cell 39, 1984, pp. 79–87).

Meijlink et al. (Proc. Natl. Acad. Sci, USA vol. 82, 1985, pp. 4987–4991).

Masui et al. (Proc. Natl. Acad. Sci. USA vol. 83, pp. 2438–2442).

Livneh et al. (J. Biol. Chem. 1986, vol. 261, pp. 12, 490–12, 497).

Rosenthal et al. (Cell, vol. 46, pp. 301–309, 1986).

Prywes et al. (EMBO vol. 5, No. 9, pp. 2179–2190, 1986).

Chou et al. (J. Biol. Chem., vol. 262, No. 4, pp. 1842–1847, 1987).

Honegger et al. (Mol. Cell. Bio. Vol. 7 No. 12, pp. 4568–4571, 1987).

Primary Examiner—David Saunders
Attorney, Agent, or Firm—Kenyon & Kenyon

[57] ABSTRACT

Inhibitors and activators of a protein whose expression affects a phenotypic characteristic of the cell, especially a cultural or morphological characteristic, are identified by their more pronounced effect on cells producing higher, usually non-naturally occurring, levels of the protein, than on cells producing little or none of the protein. In a preferred assay, the effect is observable with the naked eye.

By this method, tamoxifen is identified as an inhibitor of PKC activity in cell culture.

16 Claims, 22 Drawing Sheets

FIG. 1.A.- 1

```
          10          20          30          40          50          60
          *           *           *           *           *           *
GAA TTC CGC CTC TCC GGG CTT ACA GCC CGC GGT CCC GCC GCC CCG GGG CCG CCA CCT CTC

          70          80          90          100         110         120
          *           *           *           *           *           *
GGG GCT CCC CCC AGT CCC CGC GCG CGC AAG ATG GCT GAC CCG GCT GCG GGG CCG CCG CCG
                                    Met Ala Asp Pro Ala Ala Gly Pro Pro Pro

         130         140         150         160         170         180
          *           *           *           *           *           *
AGC GAG GGC GAG GAG AGC ACG GTG CGC TTC GCC CGC AAA GGG CCC CTC CGG CAG AAG AAC
Ser Glu Gly Glu Glu Ser Thr Val Arg Phe Ala Arg Lys Gly Pro Leu Arg Gln Lys Asn
```

FIG. 1.A. - 2

```
        190         200         210         220         230         240
         *           *           *           *           *           *
GTG CAC GAG GTG AAG AAC CAC AAA TTC ACC GCC CGC TTC TTC AAG CAG CCC ACC TTC TGC
Val His Glu Val Lys Asn His Lys Phe Thr Ala Arg Phe Phe Lys Gln Pro Thr Phe Cys

        250         260         270         280         290         300
         *           *           *           *           *           *
AGC CAC TGC ACC GAC TTC ATT TGG GGC TTC GGG AAG CAG GGA TTC CAG TGT CAA GTC TGC
Ser His Cys Thr Asp Phe Ile Trp Gly Phe Gly Lys Gln Gly Phe Gln Cys Gln Val Cys

        310         320         330         340         350         360
         *           *           *           *           *           *
TGC TTT GTT GTA CAC AAG CGC TGC CAT GAA TTC GTC ACG TTC TCC TGC CCT GGT GCA GAC
Cys Phe Val Val His Lys Arg Cys His Glu Phe Val Thr Phe Ser Cys Pro Gly Ala Asp
```

FIG. 1.A. - 3

```
          370         380         390         400         410         420
           *           *           *           *           *           *
AAG GGC CCG GCC TCT GAT GAC CCA CGG AGC AAA CAC AAG TTT AAG ATC CAC ACC TAC TCC
Lys Gly Pro Ala Ser Asp Asp Pro Arg Ser Lys His Lys Phe Lys Ile His Thr Tyr Ser

          430         440         450         460         470         480
           *           *           *           *           *           *
AGC CCT ACC TTC TGT GAC CAC TGT GGA TCA CTG CTG TAT GGG CTC ATC CAC CAG GGG ATG
Ser Pro Thr Phe Cys Asp His Cys Gly Ser Leu Leu Tyr Gly Leu Ile His Gln Gly Met

          490         500         510         520         530         540
           *           *           *           *           *           *
AAA TGC GAC ACC TGT ATG ATG AAT GTC CAC AAG CGC TGC GTG ATG AAC GTC CCC AGC CTC
Lys Cys Asp Thr Cys Met Met Asn Val His Lys Arg Cys Val Met Asn Val Pro Ser Leu
```

FIG. 1.A. - 4

```
          550         560         570         580         590         600
           *           *           *           *           *           *
TGT GGC ACC GAC CAC ACA GAA CGC CGT GGC CGC ATC TAC ATC CAG GCC CAC ATC GAC AGG
Cys Gly Thr Asp His Thr Glu Arg Arg Gly Arg Ile Tyr Ile Gln Ala His Ile Asp Arg

          610         620         630         640         650         660
           *           *           *           *           *           *
GAG GTC CTC ATC GTT GTT GTA AGA GAT GCT AAA AAT CTG GTA CCT ATG GAC CCC AAC GGC
Glu Val Leu Ile Val Val Val Arg Asp Ala Lys Asn Leu Val Pro Met Asp Pro Asn Gly

          670         680         690         700         710         720
           *           *           *           *           *           *
TTG TCA GAT CCC TAC GTA AAA CTG AAA CTG ATC CCT GAT CCC AAA AGT GAG AGC AAG CAG
Leu Ser Asp Pro Tyr Val Lys Leu Lys Leu Ile Pro Asp Pro Lys Ser Glu Ser Lys Gln
```

FIG. 1.A. - 5

```
        730         740         750         760         770         780
         *           *           *           *           *           *
AAG ACC AAG ACT ATC AAA TGC TCC CTC AAC CCG GAG TGG AAC GAA ACC TTC AGA TTT CAG
Lys Thr Lys Thr Ile Lys Cys Ser Leu Asn Pro Glu Trp Asn Glu Thr Phe Arg Phe Gln

        790         800         810         820         830         840
         *           *           *           *           *           *
CTG AAG GAA TCA GAC AAA GAC AGA AGA CTG TCC GTA GAG ATC TGG GAT TGG GAC CTG ACC
Leu Lys Glu Ser Asp Lys Asp Arg Arg Leu Ser Val Glu Ile Trp Asp Trp Asp Leu Thr

        850         860         870         880         890         900
         *           *           *           *           *           *
AGC AGG AAT GAC TTC ATG GGA TCT CTG TCG TTT GGG ATT TCA GAA CTA CAG AAA GCC GGA
Ser Arg Asn Asp Phe Met Gly Ser Leu Ser Phe Gly Ile Ser Glu Leu Gln Lys Ala Gly
```

FIG. 1.A. - 6

```
        910         920         930         940         950         960
         *           *           *           *           *           *
GTG GAT GGC TGG TTC AAG TTA CTA AGC CAG GAA GAA GGC GAG TAC TTT AAT GTG CCG GTG
Val Asp Gly Trp Phe Lys Leu Leu Ser Gln Glu Glu Gly Glu Tyr Phe Asn Val Pro Val

        970         980         990        1000        1010        1020
         *           *           *           *           *           *
CCG CCG GAA GAA AGC GAG GGC AAT GAA GAG CTG CGG CAG AAG TTT GAG AGA GCC AAG ATT
Pro Pro Glu Glu Ser Glu Gly Asn Glu Glu Leu Arg Gln Lys Phe Glu Arg Ala Lys Ile

       1030        1040        1050        1060        1070        1080
         *           *           *           *           *           *
GGC CAA GGT ACC AAG GCT CCA GAA GAA AAG ACA GCG AAC ACT ATA TCC AAA TTT GAC AAC
Gly Gln Gly Thr Lys Ala Pro Glu Glu Lys Thr Ala Asn Thr Ile Ser Lys Phe Asp Asn
```

FIG. 1.A. - 7

```
      1090        1100        1110        1120        1130        1140
       *           *           *           *           *           *
AAT GGC AAC AGG GAC CGG ATG AAA CTG ACC GAT TTT AAC TTC CTG ATG GTG CTG GGG AAA
Asn Gly Asn Arg Asp Arg Met Lys Leu Thr Asp Phe Asn Phe Leu Met Val Leu Gly Lys

      1150        1160        1170        1180        1190        1200
       *           *           *           *           *           *
GGC AGC TTT GGC AAG GTC ATG CTC TCA GAG CGG AAG GGT ACA GAT GAA CTC TAT GCC GTG
Gly Ser Phe Gly Lys Val Met Leu Ser Glu Arg Lys Gly Thr Asp Glu Leu Tyr Ala Val

      1210        1220        1230        1240        1250        1260
       *           *           *           *           *           *
AAG ATC CTG AAG AAA GAT GTG GTG ATC CAA GAT GAC GAT GTG GAG TGC ACA ATG GTG GAG
Lys Ile Leu Lys Lys Asp Val Val Ile Gln Asp Asp Asp Val Glu Cys Thr Met Val Glu
```

FIG. 1.A. - 8

```
      1270        1280        1290        1300        1310        1320
        *           *           *           *           *           *
AAG AGG GTG CTG GCC CTG CCT GGG AAG CCC CCA TTC CTG ACT CAG CTC CAT TCC TGC TTC
Lys Arg Val Leu Ala Leu Pro Gly Lys Pro Pro Phe Leu Thr Gln Leu His Ser Cys Phe

      1330        1340        1350        1360        1370        1380
        *           *           *           *           *           *
CAG ACC ATG GAC CGC CTC TAC TTT GTG ATG GAG TAT GTG AAC GGG GGC GAC CTC ATG TAC
Gln Thr Met Asp Arg Leu Tyr Phe Val Met Glu Tyr Val Asn Gly Gly Asp Leu Met Tyr

      1390        1400        1410        1420        1430        1440
        *           *           *           *           *           *
CAC ATC CAA CAA GTT GGC CGT TTC AAG GAG CCC CAT GCT GTA TTT TAC GCT GCA GAG ATT
His Ile Gln Gln Val Gly Arg Phe Lys Glu Pro His Ala Val Phe Tyr Ala Ala Glu Ile
```

FIG. 1.A. - 9

```
      1450        1460        1470        1480        1490        1500
       *           *           *           *           *           *
GCC ATC GGT CTT TTC TTC TTG CAG AGC AAG GGC ATC ATT TAC CGT GAC CTG AAA CTT GAC
Ala Ile Gly Leu Phe Phe Leu Gln Ser Lys Gly Ile Ile Tyr Arg Asp Leu Lys Leu Asp

      1510        1520        1530        1540        1550        1560
       *           *           *           *           *           *
AAC GTG ATG CTG GAT TCC GAG GGG CAC ATC AAA ATC GCT GAC TTT GGC ATG TGT AAA GAG
Asn Val Met Leu Asp Ser Glu Gly His Ile Lys Ile Ala Asp Phe Gly Met Cys Lys Glu

      1570        1580        1590        1600        1610        1620
       *           *           *           *           *           *
AAT ATC TGG GAT GGG GTG ACA ACC AAG ACA TTC TGT GGC ACT CCA GAC TAC ATT GCC CCA
Asn Ile Trp Asp Gly Val Thr Thr Lys Thr Phe Cys Gly Thr Pro Asp Tyr Ile Ala Pro
```

FIG. 1.A. - 10

```
      1630        1640        1650        1660        1670        1680
       *           *           *           *           *           *
GAG ATC ATT GCT TAT CAG CCC TAC GGA AAG TCT GTG GAC TGG TGG GCG TTT GGA GTC CTG
Glu Ile Ile Ala Tyr Gln Pro Tyr Gly Lys Ser Val Asp Trp Trp Ala Phe Gly Val Leu

      1690        1700        1710        1720        1730        1740
       *           *           *           *           *           *
CTG TAT GAA ATG TTG GCT GGC CAG GCA CCT TTT GAA GGG GAG GAT GAG GAT GAA CTC TTC
Leu Tyr Glu Met Leu Ala Gly Gln Ala Pro Phe Glu Gly Glu Asp Glu Asp Glu Leu Phe

      1750        1760        1770        1780        1790        1800
       *           *           *           *           *           *
CAG TCA ATC ATG GAG CAC AAC GTG GCG TAT CCC AAG TCC ATG TCT AAG GAA GCT GTG GCA
Gln Ser Ile Met Glu His Asn Val Ala Tyr Pro Lys Ser Met Ser Lys Glu Ala Val Ala
```

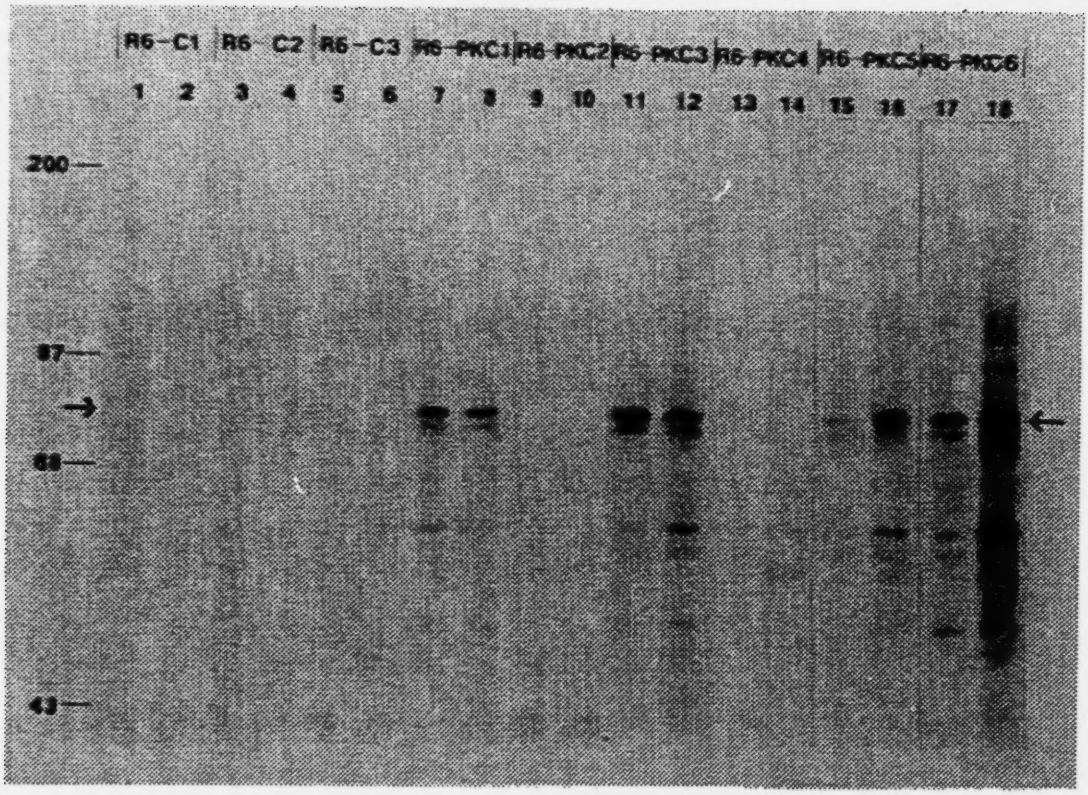
FIG. 1.A. - 11

```
      1810        1820        1830        1840        1850        1860
       *           *           *           *           *           *
ATC TGC AAA GGG CTA ATG ACC AAA CAC CCA GGC AAG CGC CTG GGT TGT GGG CCT GAA GGG
Ile Cys Lys Gly Leu Met Thr Lys His Pro Gly Lys Arg Leu Gly Cys Gly Pro Glu Gly

      1870        1880        1890        1900        1910        1920
       *           *           *           *           *           *
GAA CGA GAC ATT AAG GAG CAT GCA TTT TTC CGG TAT ATC GAC TGG GAG AAA CTC GAA CGC
Glu Arg Asp Ile Lys Glu His Ala Phe Phe Arg Tyr Ile Asp Trp Glu Lys Leu Glu Arg

      1930        1940        1950        1960        1970        1980
       *           *           *           *           *           *
AAG GAG ATT CAG CCA CCT TAT AAA CCA AAA GCT AGA GAC AAG CGA GAC ACC TCC AAC TTC
Lys Glu Ile Gln Pro Pro Tyr Lys Pro Lys Ala Arg Asp Lys Arg Asp Thr Ser Asn Phe
```

FIG. 1.A. - 12

```
        1990          2000          2010          2020          2030          2040
          *             *             *             *             *             *
GAC AAA GAG TTC ACC AGG CAG CCT GTG GAA CTG ACT CCC ACT GAC AAA CTC TTC ATC ATG
Asp Lys Glu Phe Thr Arg Gln Pro Val Glu Leu Thr Pro Thr Asp Lys Leu Phe Ile Met

        2050          2060          2070          2080          2090          2100
          *             *             *             *             *             *
AAC TTG GAC CAA AAT GAA TTT GCT GGC TTC TCG TAT ACT AAC CCA GAG TTT GTC ATT AAT
Asn Leu Asp Gln Asn Glu Phe Ala Gly Phe Ser Tyr Thr Asn Pro Glu Phe Val Ile Asn

        2110          2120          2130          2140          2150          2160
          *             *             *             *             *             *
GTG TAG GTG AAT GCA GAT TCC ATC GCT GAG CCT GTG TGT AAG GCT GCA GCG TGA ATG TCT
Val ---
```

FIG. 2



BEST AVAILABLE COPY

FIG. 3

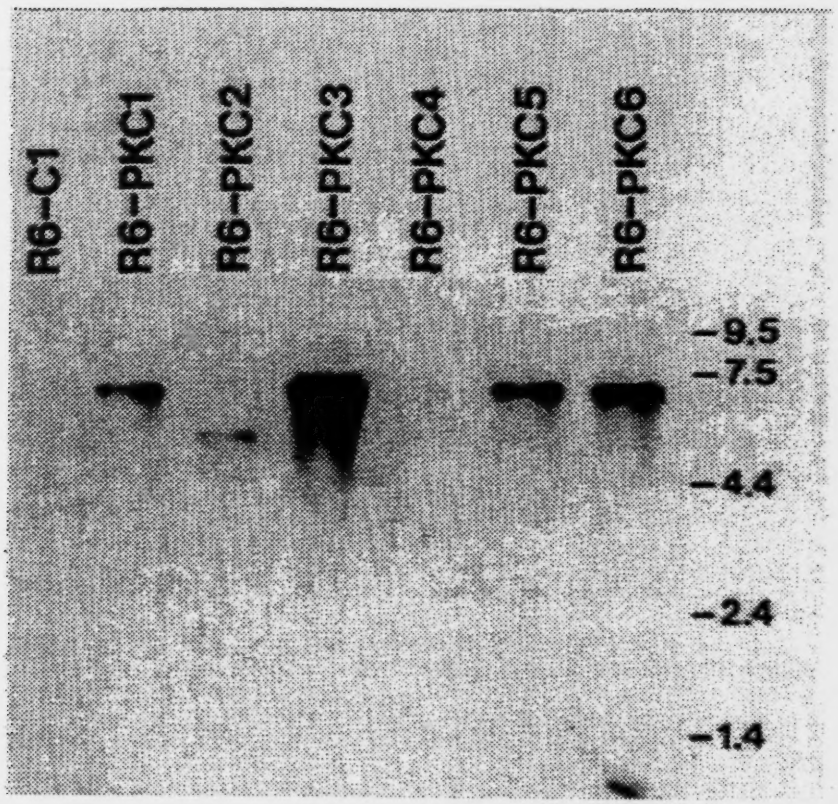

R6-C1
R6-PKC1
R6-PKC2
R6-PKC3
R6-PKC4
R6-PKC5
R6-PKC6
-9.5
-7.5
-4.4
-2.4
-1.4

FIG. 4

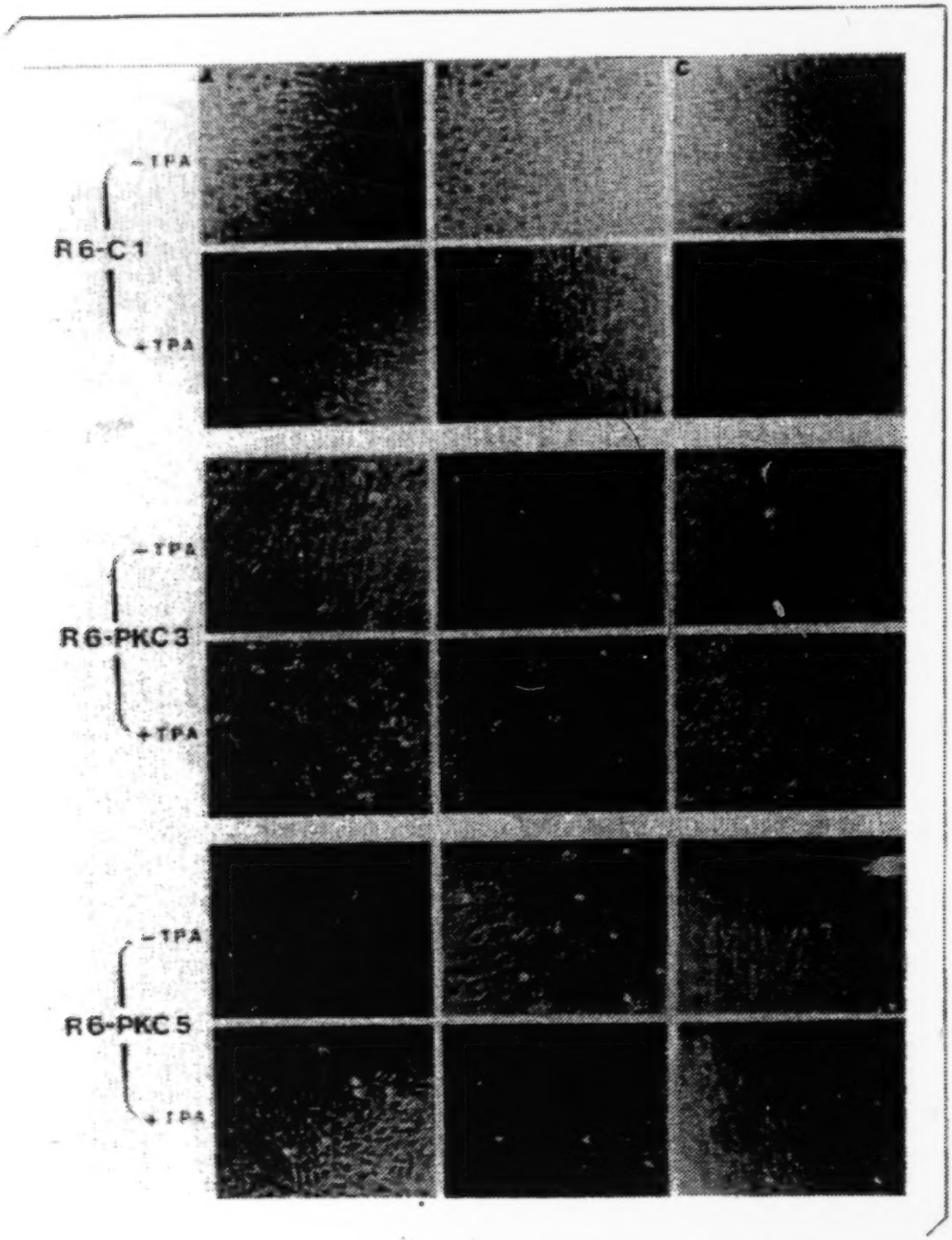


FIG. 5.

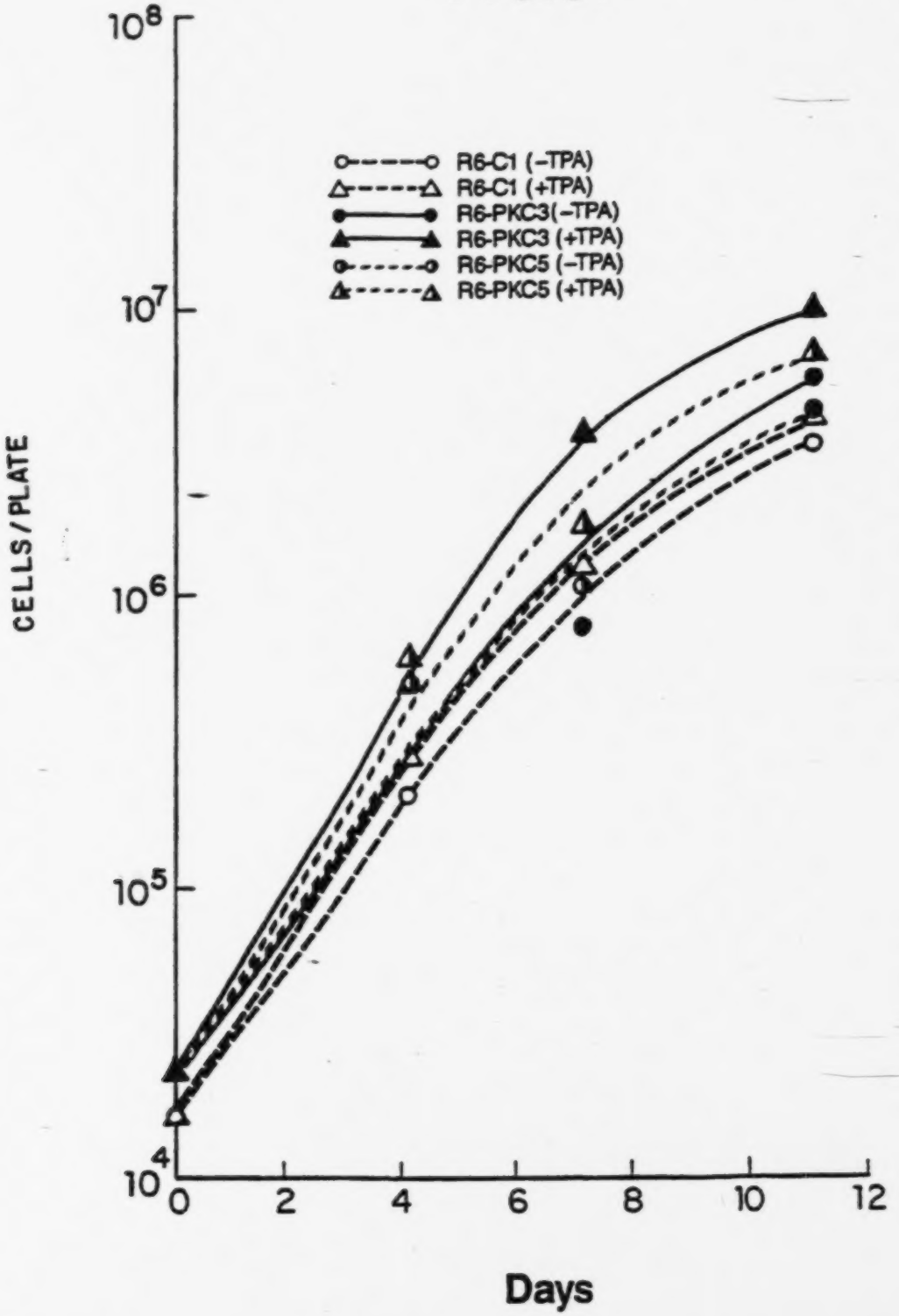

R6-C1 (-TPA)
R6-C1 (+TPA)
R6-PKC3(-TPA)
R6-PKC3 (+TPA)
R6-PKC5 (-TPA)
R6-PKC5 (+TPA)
CELLS/PLATE
10^8
10^7
10^6
10^5
10^4
0
2
4
6
8
10
12
Days

FIG. 6

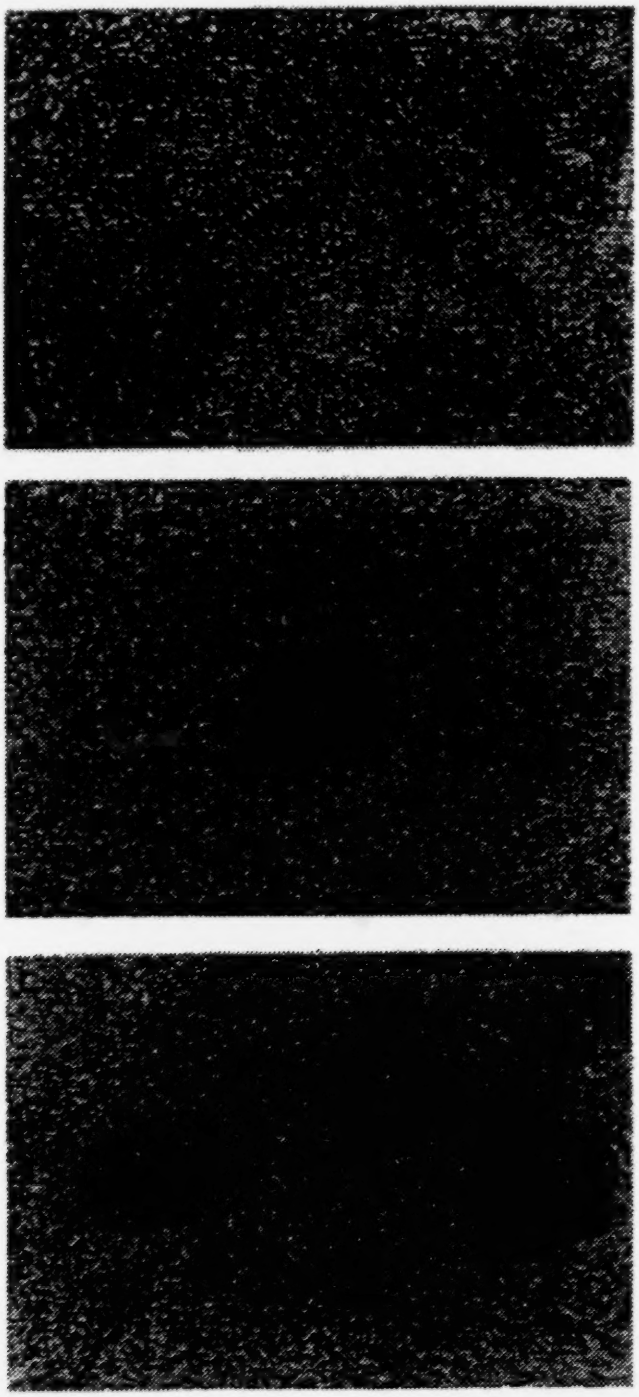


FIG. 7

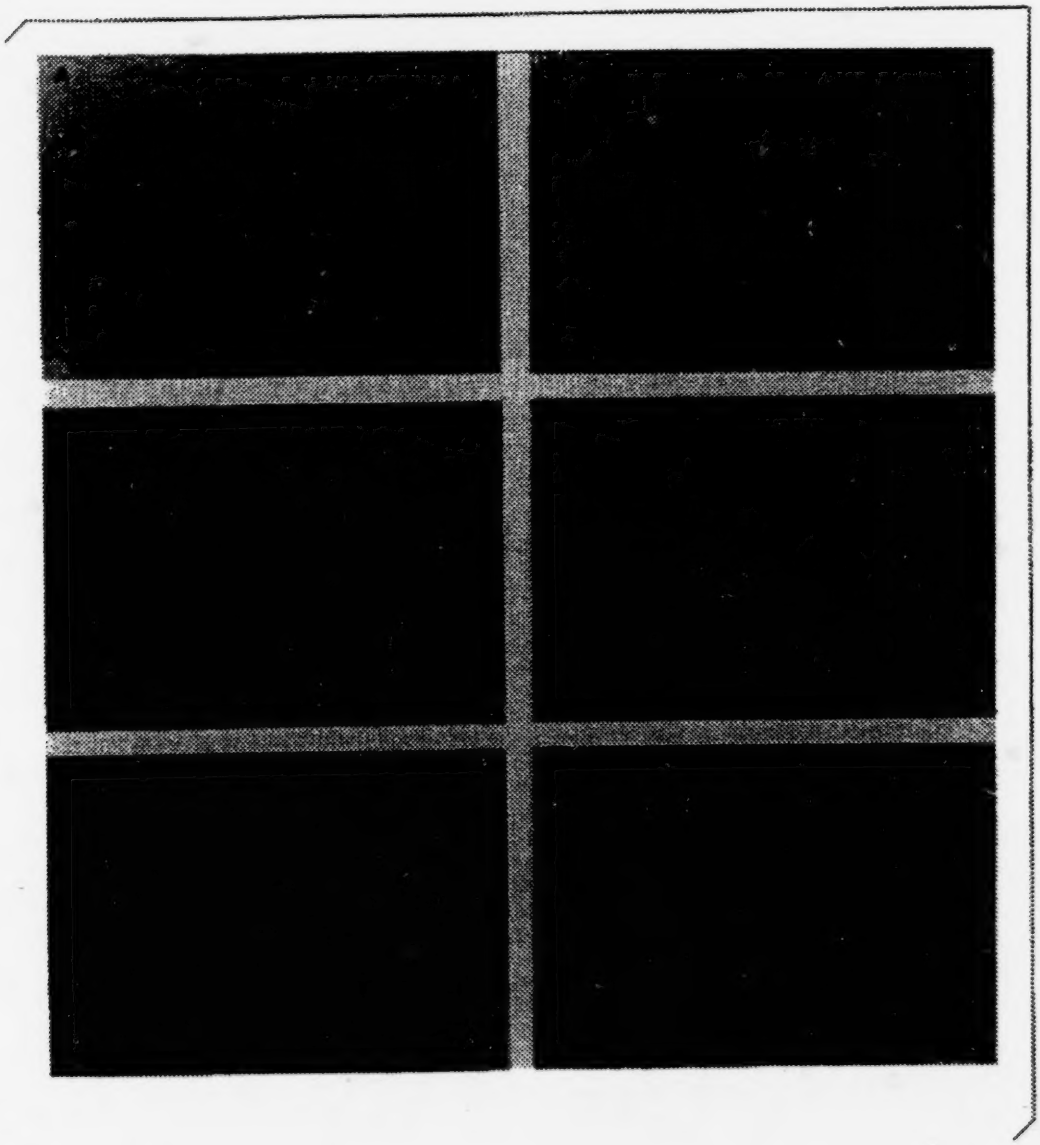


FIG. A.1. - 13

```
      2170        2180        2190        2200        2210        2220
       *           *           *           *           *           *
ATT ATC AAT TCC AGT CTT CCA GGA TTC ATG GTG CCT CTG TTG GCA TCC GTC ATG TGG AGA

      2230        2240        2250        2260        2270        2280
       *           *           *           *           *           *
GCT TGT CTT AGA GGG CTT TTC TTT GTA TGT ATA GCT TGC TAG TTT GTT TTC TAC ATT TCA

      2290        2300        2310        2320        2330        2340
       *           *           *           *           *           *
AAA TGT TTA GTT TAG AAT AAG TGC ATT GCC CAC TGA TAG AGG TAC AAT TTT CCA GAC TTC
```

FIG. 1.A. - 14

```
        2350        2360        2370        2380        2390        2400
         *           *           *           *           *           *
CAG AAA CTC ATC CAA TGA ACC AAC AGT GTC AAA ACT TAA CTG TGT CCG ATA CCA AAA TGC

        2410        2420        2430        2440        2450        2460
         *           *           *           *           *           *
TTC AGT ATT TGT AAT TTT TAA AGT CAG ATG CTG ATG TTC CTG GTC AAA GTT TTT ACA GTT

        2470        2480        2490        2500        2510        2520
         *           *           *           *           *           *
ACT CTC GAA TAT CTC CTT TGA ATG CTA CCT AAG CAT GAC CGG TAT TTT TAA AAG TTG TGA
```

FIG. 1.A. - 15

```
          2530          2540          2550          2560          2570          2580
            *             *             *             *             *             *
GTA AGC TTT GCA GTT ACT GTG AAC TCT TGT CTC TTG GAG GAA CTT TTT GTT TAA GAA TTG

          2590          2600
            *             *
GTA TGA TTA AAC TGA ATT C*
```

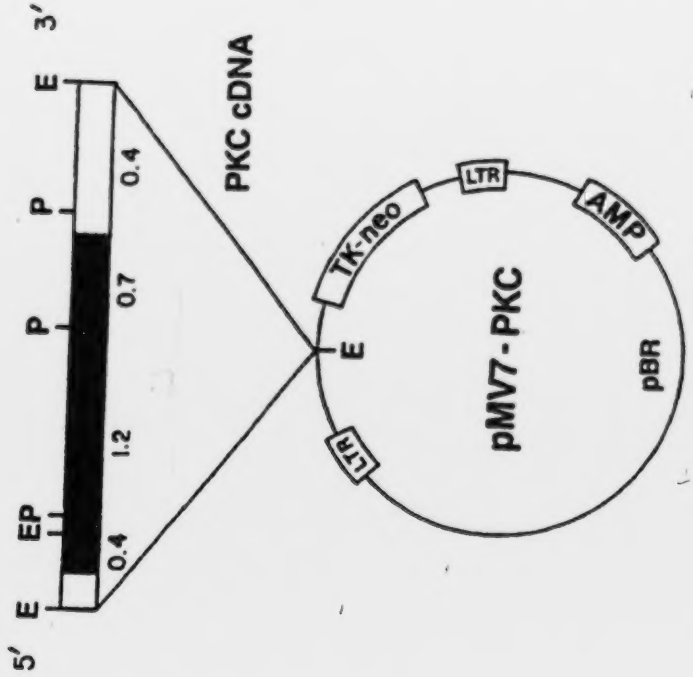

5'
E
EP
P
P
E
3'
0.4
1.2
0.7
0.4
PKC cDNA
TK-neo
LTR
AMP
pMV7-PKC
pBR
LTR
E

FIG. 1.
B.

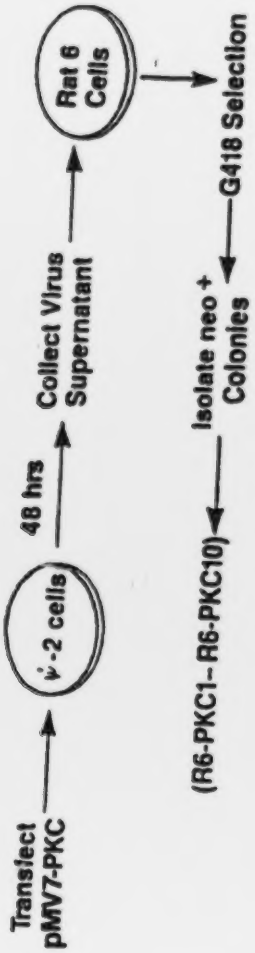

Transfect pMV7-PKC
ψ-2 cells
48 hrs
Collect Virus Supernatant
Rat 6 Cells
G418 Selection
Isolate neo + Colonies
(R6-PKC1- R6-PKC10)

FIG. 1.
C.

1

METHOD OF SCREENING FOR PROTEIN INHIBITORS AND ACTIVATORS

This application is a continuation of Ser. No. 08/977,986, filed Nov. 18, 1992 and now abandoned which in turn is a a continuation of application Ser. No. 07/392,073, filed on Aug. 10, 1989 now U.S. Pat. No. 5,266,464 which in turn is a continuation-in-part of U.S. Ser. No. 07/154,206, filed Feb. 10, 1988, now U.S. Pat. No. 4,980,281 incorporated by reference herein, the benefit of whose filing date is claimed pursuant to 35 U.S.C. Sec. 120.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates to a general screening method for the discovery and identification of both inhibitors and activators of enzymes, receptors, and other proteins. In particular, it is concerned with a method of screening for substances which specifically inhibit or activate a particular protein affecting the cultural or morphological characteristics of the cell expressing the protein, especially in a manner apparent to the naked eye.

INFORMATION DISCLOSURE STATEMENT

A number of assay systems are currently in use for the discovery of new modulators of cell growth, and in particular, in the search for new anti-cancer drugs which are specifically toxic to cancer cells but not to normal cells. A variety of changes may be scored for, but the most common ones are reversion of the transformed phenotype, significant changes in cell morphology, or cytotoxicity. The assays include: (1) in vitro cytotoxicity assays; (2) soft agar colony formation assays; (3) in vitro anti-microbial assays; and (4) assays which detect changes in cellular morphology.

In vitro cytotoxicity assays involve the measurement of cellular parameters which are indicative of inhibition of cellular growth or cytotoxicity. These include, for example, the measurement of the inhibition of certain cellular metabolic pathways in response to treatment with cytotoxic agents. The papers by Von Hoff, et al. (1985), and Catino, et al. (1985) describe typical methods which use this technique. These methods are somewhat complex technically, and require the use of radioactive tracers in some cases. Furthermore, the results are non-specific since any agent which alters the growth properties of cells will score positively in these assay systems.

Agents have also been tested for their ability to inhibit transformed (cancerous) cells from growing in soft agar. This method is based upon the finding by Freedman and Shin (1974) that the formation of colonies of cells in soft agar is the in vitro test which shows the highest correlation in predicting whether the cells will be tumorigenic in an experimental animal. This method is relatively simple to perform since colony growth will, after two or more weeks, generally be large enough to be seen with the naked eye. Scoring the final results, therefore, can be performed either by a technician without extensive training in tissue culture, or, as we describe in the current application, by an automated absorbance detection system. In its present form, however, this method is also non-specific for the same reasons as described above. In other words, any agent which inhibits cellular growth in any way will scores positively in this assay system as it is currently used, whether or not it inhibits the protein of interest.

In vitro anti-microbial assays involve the use of bacterial or yeast strains which are used as test organisms for screening for agents with generalized growth inhibitory properties (also described in Catino, et al., 1985). In this method, the bacterial or yeast strain is grown on standard media plates and potential agents are applied to various spots on the plates. If an agent has growth inhibitory properties, a clear zone results at the site of its application on the plate, resulting from the inability of the test strain to grow in the area. This method is rapid and can be performed by a technician without extensive training in tissue culture techniques, but the results are generally non-specific because agents which are effective against bacterial or yeast strains are frequently less effective (or completely ineffective) in modulating the growth of mammalian cells, as shown in the paper by Catino et al. (1985).

Still other screening systems depend upon a morphologic alteration of the test cells by exposure to the potential agents in order to determine the effectiveness of a given agent. This method is currently the most effective one for developing specific agents which interact with a given protein or alter a specific cellular property, as evidenced by the representative paper by Uehara, et. al. (1985). However, these screening systems are the most difficult ones to apply in practice, since the morphologic effect of each individual agent on the test cells must be studied under the microscope. Hence this method requires extensive observations of the cells by a trained scientist.

SUMMARY OF THE INVENTION

The Method presented in detail in this application combines the rapidity and ease of performance of the soft agar assay with a specificity for detecting an active agent exceeding that of the morphology assay. In brief, the method which we describe herein involves the generation of a cell line purposefully engineered to detect both stimulatory and inhibitory agents which are absolutely specific for any given protein which affects the cultural or morphological characteristics of the cell.

The basis for this invention is my observation that if a protein (the "protein of interest", or POI) which is involved in some manner in cellular growth control is overproduced in cells, then pharmacologic agents which can activate or inhibit the POI can result in altered growth properties of the cells.

The sensitivity of the cells is dependent on their production of the POI, a phenomenon referred to herein as a "graded cellular response" to the pharmacologically active agent.

The present invention provides a rapid, yet powerful screening system for the discovery and identification of both inhibitors and activators of proteins. The method may be applied to virtually any type of protein, including enzymes, receptors, DNA- or RNA-binding proteins, or others which are directly or indirectly involved in regulating cellular growth.

The method involves the insertion of a DNA (or cDNA) sequence encoding the Protein Of Interest (POI) into an appropriate vector and the generation of cell lines which contain either (1) the expression vector alone ("control" cell lines) or (2) the expression vector containing the inserted DNA (or cDNA) sequence encoding the POI ("test" cell lines). Using the appropriate vector system, recipient cell lines, and growth conditions, test cell lines can thus be generated which stably overproduce the corresponding POI. Under the appropriate growth conditions, these cell lines will exhibit a "graded cellular response" to activators or inhibitors of the POI. A screening system can thus be set up

whereby the control and test cell lines are propagated in defined growth conditions in tissue culture dishes (or even in experimental animals) and large numbers of compounds (or crude substances which may contain active compounds) can be screened for their effects on the POI.

Substances which inhibit or activate the POI may affect characteristics such as growth rate, tumorigenic potential, anti-tumorigenic potential, anti-metastatic potential, cell morphology, antigen expression, and/or anchorage-independent growth capability. Substances which specifically inhibit or inactivate the POI may be distinguished from substances which affect cell morphology or growth by other mechanisms in that they will have a greater effect on the test lines than on the control lines.

The system has been tested using several cDNA sequences and several recipient cell lines, and can be easily automated.

The appended claims are hereby incorporated by reference as an enumeration of the preferred embodiments. All references cited anywhere in this specification are hereby incorporated by reference to the extent pertinent.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows the full-length cDNA sequence, and the deduced amino acid sequence, of one of several forms of PKC which has previously been isolated (cDNA clone RP58), and whose partial sequence has been reported (Housey, et al., 1987). It corresponds to PKCbeta1 according to the nomenclature of Ono, et al. (1987). The deduced amino acid sequence begins with the first in-frame methionine codon at nucleotide position 91 and encodes a 671 amino acid protein with a predicted molecular weight of 76.8 kd. A consensus polyadenylation signal is underlined.

FIG. 1B shows the retrovirus-derived cDNA expression vector, developed in this laboratory, which was used for the present studies. The full-length RP58 cDNA encoding PKC-beta1 (shown in 1A) was cloned into the Eco RI site of plasmid pMV7. The shaded region represents the coding sequence. "E" and "P" designate Eco RI and Pst I restriction sites, respectively. The indicated sizes between restriction sites in the RP58 cDNA are given in kilobases. "LTR" designates the 5' (left) and 3' (right) long terminal repeats of Moloney murine leukemia virus, and "TK-neo" designates the promoter region of the HSV thymidine kinase gene linked to the 5' end of the bacterial neomycin phosphotransferase (neo) gene.

FIG. 1-C outlines in schematic form the overall strategy used to generate cell lines stably overproducing PKC.

FIG. 2. Purification and Autophosphorylation of PKC. PKC activity from each cell line was purified and subjected to reaction conditions favoring autophosphorylation of PKC. Following the autophosphorylation reaction, protein samples were separated by discontinuous polyacrylamide gel electrophoresis. In the lanes bearing odd numbers the reaction mixtures contained 1 mM Ca2+ and phosphatidyl serine to activate PKC, and in the lanes bearing even numbers the reaction mixtures contained 1 mM EGTA, 100 ng/ml TPA, and phosphatidylserine. The numbers in the left margin indicate the sizes of molecular weight markers, in kd. Arrows indicate the position of the 75 kd autophosphorylated PKC.

FIG. 3. Northern Blot Hybridization Analyses. Poly A+ RNA was isolated from the indicated cell lines and separated by electrophoresis on 6% formaldehyde/1% agarose gels, blotted onto nylon membrane and hybridized with the 32P-labelled full-length RP58 cDNA probe, as previously described (Housey, et. al., 1987). The numbers in the right margin indicate the sizes in kb of the RNA markers. The R6-PKC4 sample displayed a very weak 4.8 kb band on the original autoradiograph.

FIG. 4. Morphologic Responses of the Cell Lines to Phorbol Ester Treatment. Nearly confluent cultures of the three indicated cell lines were exposed to 100 ng/ml TPA in 0.1% dimethylsulfoxide (DMSO) solvent ("+TPA") or 0.1% DMSO alone ("−TPA"), in DMEM plus 10% CS. Photographs were taken 24 hours later (Panels A and D) and 48 hours later (Panels B and E). Fresh medium plus or minus TPA was then added and photographs were then taken an additional 24 hours later (Panels C and F). (Magnification: 100×).

FIG. 5. Growth Curves of Control and PKC-Overproducing Cell Lines. The indicated cell lines were seeded at 1×10^4 per 6 cm plate in DMEM plus 10% CS, in the presence ("+TPA") and absence ("−TPA") of 100 ng/ml TPA. Cell numbers were determined in replicate plates during the subsequent 11 day growth period. The values given indicate the means of triplicate determinations, which varied by less than 10%.

FIG. 6. Post-confluence Foci Formation. Control R6-C2 cells (Panel A) and R6-PKC3 cells (Panels B And C) were grown to confluence and then maintained for an additional 28 days in DMEM plus 10% CS (without TPA), with the addition of fresh medium every 3 days. Photographs were taken at the end of the 28 day period. Magnification: 40×.

FIG. 7. Growth in Soft Agar. Cell lines R6-C1, R6-PKC3, and R6-PKC5 were seeded into 60 mm petri dishes in 0.3% agar containing DMEM plus 20% FBS and 50 lg/ml G418, plus or minus 100 ng/ml TPA. Photographs were taken after 21 days of growth. A) R6-C1+TPA (low-power field) B) R6-C1+TPA (medium-power field)C) R6-PKC3- D) R6-PKC3+TPA E) R6-PKC5 F) R6-PKC5+TPA For additional details see Experimental Procedures. (Magnification : 100× in panel B; 40× in all other panels).

DETAILED DESCRIPTION OF THE INVENTION

The present method is intended for use in identifying potential chemical inhibitors or activators of enzymes, receptors, or any proteins which have effects upon cell phenotype. This method requires two cell lines, preferably alike except for their expression (production) of the protein of interest at different levels (and any further differences necessitated by that difference in expression). Inhibitors or activators are identified by their greater effect on the phenotype of the higher producing cell line.

Any phenotypic characteristic of the cell which is affected by expression of the protein of interest, other, of course, than the level of the protein itself, may be assayed. The phenotypic characteristic is preferably a "cultural" or "morphological" characteristic of the cell. For purposes of the appended claims, these terms are defined as follows:

Cultural characteristics include, but are not limited to the nutrients required for growth, the nutrients which, though not required for growth, markedly promote growth, the physical conditions (temperature, pH, gaseous environment, osmotic state, and anchorage dependence or independence) of the culture which affect growth, and the substances which inhibit growth or even kill the cells.

Morphological characteristics, but are not limited to include the size and shape of cells, their arrangements, cell differentiation, and subcellular structures.

Where the protein of interest is implicated in tumorigenesis or related phenomena, the characteristic observed is

preferably one related to cellular growth control, differentiation, de-differentiation, carcinogenic transformation, metastasis, tumorigenesis, or angiogenesis.

Phenotypic changes which are observable with the naked eye are of special interest. Changes in the ability of the cells to grow in an anchorage-independent manner, to grow in soft agar, to form foci in cell culture, and to take up selected stains are particularly appropriate phenomena for observation and comparison.

The higher producing cell line is preferably obtained by introducing a gene encoding the Protein of Interest (POI) into a host cell. The gene may be a one isolated from the genome of an organism, a cDNA prepared from an mRNA transcript isolated from an organism, or a synthetic duplicate of a naturally occurring gene. It may also have a sequence which does not occur exactly in nature, but rather corresponds to a mutation (single or multiple) of a naturally occurring sequence. No limitation is intended on the manner in which this mutated sequence is obtained. The gene is operably linked to a promoter of gene expression which is functional in the host, such that the corresponding Protein Of Interest (POI) is stably "overproduced" in the recipient cells to differing degrees. The promoter may be constitutive or inducible. By "overproduced", I mean that the POI is expressed at higher levels in the genetically manipulated cell line than in the original cell line. This allows one to generate cell lines which contain (or secrete) from as little as a few fold to more than 100-fold elevated levels of the POI relative to the control cells.

Any method may be used to introduce the gene into the host cell, including transfection with a retroviral vector, direct transfection (e.g., mediated by calcium phosphate or DEAE-dextran), and electroporation. Preferably, a retroviral vector is used.

The host cells should exhibit a readily observable phenotypic change as a result of enhanced production of the POI. Preferably, this response should be proportional to the level of production of the POI. Finally, the cells should not spontaneously manifest the desired phenotypic change. For example, 3T3 cells form foci spontaneously. Among the preferred cell lines are Rat-6 fibroblasts, C3H10T ½ fibroblasts, and HL60. (HL60 is a human cell line that differentiates in response to PKC activation.) 3T3 cells may be used, but with the reservation stated above.

Generally speaking, it is preferable to maximize the ratio of production by the "overproducing" cell line to production by the "native" line. This is facilitated by selecting a host cell line which produces little or no POI, and introducing multiple gene copies and/or using high signal strength promoters.

The Rat 6 embryo fibroblast cell line is a variant of the rat embryo fibroblast cell line established by Freeman et. al., (1972) and isolated by Hsiao et al., 1986. This cell line has an unusually flat morphology, even when maintained in culture at post-confluence for extended periods of time, displays anchorage dependent growth and, thus far, has not undergone spontaneous transformation. It was also ideal for these studies since it has a very low level of endogenous PKC activity and a low level of high affinity receptors for phorbol esters.

While my-most preferred host cell line is the Rat-6 fibroblast cell line, I have tested this Method with other cell types, including the mouse NIH-3T3 cell line as well as the C3H 10T1/2 cell line. Tables 1(a) and (b) below show the representative specific activities of seven NIH-3T3 and six C3H-10T1/2 cell lines stably overproducing PKC. I have also recently performed the same experiments with the human HeLa cell line. In each case the resulting cell lines all exhibited growth properties qualitatively identical to those described for the PKC-expressing Rat-6 fibroblast cell lines. More specifically, C3H 10T1/2 and NIH 3T3 cells which overproduced PKC exhibited increased cell density, refractility, and rounding relative to the "native" lines. The PKC-overproducing NIH 3T3 cells also displayed increased foci formation. PKC-overproducing C3H 10T1/2 cells also formed foci, but only when exposed to a phorbol ester, i.e., a PKC activator. These results clearly demonstrate that many different types of cells can be employed in this method. The experimental procedures used to generate these cell lines were also identical to those used in connection with the Rat-6 cell line.

If a cell line otherwise suitable for use as a control cell line produces excessive POI, it is possible to inhibit this production by incorporation a known inhibitor into the culture medium for both the control and test cell lines, thus achieving a more favorable ratio of production. Contrawise, if the level of POI production by the test cell line is too low, a known activator may be incorporated into the culture media.

It is desirable, but not necessary, that a suspected inhibitor or activator be tested on both a control line and an overproducing line in parallel.

What we are looking for is a increase in the phenotypic change exhibited by the cell which becomes greater with increasing expression of the POI. We call this a "graded cellular response," and it is by this specialized response that we distinguish inhibitors or activators of the POI from agents that act upon other cell metabolites to effect a phenotypic change.

Thus, in a preferred embodiment, the cell lines are assayed for their relative levels of the POI, and their ability to grow in anchorage-independent systems (e.g., matrices such as soft agar or methocel), to form small "foci" (areas of dense groups of cells clustered together and growing on top of one another) in tissue culture dishes, to take up selected stains, or to bind an appropriately labeled antibody or other receptor for a cell surface epitope. In addition to exhibiting these growth control abnormalities, such cell lines will also be sensitive in their growth properties to chemical agents which are capable of binding to, or modifying the biological effects of, the POI.

The method is particularly unique in that it can be employed to search rapidly for EITHER activators OR inhibitors of a given POI, depending upon the need. The term "activators," as used herein, includes both substances necessary for the POI to become active in the first place, and substance which merely accentuate its activity. The term "inhibitors" incindes both substance which reduce the activity of the POI and these which nullify it altogether. When a POI has more than one possible activity. The inhibitor or activator may modulate any or all of its activities.

The use of this screening method to identify inhibitors or activators of enzymes is of special interest. In particular, I am interested in using it to identify inhibitors or activators of eazymes involved in tumorigenesis and related phenomena, for example, protein kinase C, ornithine decarboxylase, cyclic AMP-dependent protein kinase, the protein kinase domains of the insulin and EGF receptors, and the enzyme products of various cellular onc genes such as the c-src ($PP60^{src}$) or c-ras ($P20^{ras}$) genes.

The present invention may be used to identify activators or inhibitors of receptor proteins, both cytoplasmic receptors and integral membrane receptors, provided that overproduc-

tion of the receptor protein has a detectable effect on the producing cells.

Protein kinase C (PKC) is a Ca^{2+}— and phospholipid-dependent serine/threonine protein kinase of fundamental importance in cellular growth control. PKC is activated endogenously by a wide variety of growth factors, hormones, and neurotransmitters, and has been shown to be a high affinity receptor for the phorbol ester tumor promoters as well as other agents which possess tumor promoting activity (for reviews see Nishizuka 1986; 1984; Ashendel, 1984). PKC has been shown to phosphorylate several intracellular protein substrates, including the epidermal growth factor (EGF) receptor (Hunter et al., 1984), pp60src (Gould et al., 1985), the insulin receptor (Bollag et al., 1986), p21 ras (Jeng et al., 1987), and many others (Nishizuka, 1986). Several laboratories have recently isolated cDNA clones encoding distinct forms of PKC, thus demonstrating that PKC is encoded by a multigene family (Ono et al., 1986, Knopf et al., 1986, Parker et al., 1986; Coussens et al., 1986; Makowske et al., 1986; Ohno et al., 1987; Housey et al., 1987). The multiple forms of PKC exhibit considerable tissue specificity (Knopf, et. al., 1986; Brandt et al., 1987; Ohno, et al, 1987; Housey, et. al., 1987) which suggests that there may be subtle differences in the function(s) of each of the distinct forms. However, all of the cDNA clones which have been isolated thus far that encode distinct forms of PKC share at least 65% overall deduced homology at the amino acid level, and transient expression experiments with some of these cDNA clones have shown that they encode serine/threonine protein kinase activities which bind to, or are activated by, the phorbol ester tumor promoters (Knopf, et. al., 1986, Ono, et. al., 1987).

We used the PKCbeta1 cDNA clone for the present studies for the following reasons. With the exception of the brain, where its expression is very high, PKCbeta1 is expressed at very low levels in most tissues, and its expression is virtually undetectable in Rat 6 fibroblasts (see below). Thus, we reasoned that using this form would maximize the phenotypic differences observed between control cells and cells overexpressing the introduced form of PKC. The PKCbeta1 form is also of particular interest because within the PKC gene family its deduced carboxyl terminal domain displays the highest overall homology to the catalytic subunit of the cyclic AMP-dependent protein kinase (PKAc) and the cyclic GMP-dependent protein kinase (PKG) (Housey et al., 1987). The latter observation suggests that PKAc, PKG, and the beta1 form of PKC may share a common ancestral serine/threonine protein kinase progenitor, and that the additional PKC genes may have been derived through evolutionary divergence from the beta1 form.

Agents which interact with certain structural proteins, such as actin and myosin, are also of interest. Mutations in the genes expressing these proteins may be involved in tumorigenesis and metastasization. Such interactions can lead to changes in cell phenotype which can be assayed by this method.

As is set forth in greater detail below, I have produced cell lines which overproduce protein kinase C (PKC). These cell lines, unlike the control cells, grow in soft agar even in the absence of the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA has been shown to be a potent activator of PKC. When TPA is added to the growth medium the PKC-overproducing cell lines grow even better and form considerably larger colonies in soft agar. Furthermore, I have also tested known inhibitors of PKC activity which, as predicted, caused the PKC-overproducing cells to grow less well (or not at all) in soft agar. Thus, the direct utility of this method in identifying both activators or inhibitors of a gene product, in this case PKC, has been clearly demonstrated.

In additional studies with other genes, most notably the c-H-ras oncogene, the c-myc oncogene, and certain cDNA clones encoding phorbol-ester inducible proteins, overproducing cell lines which exhibited morphological changes relative to the native lines were likewise obtained, and it is believed that such cell lines may be used in screening for inhibitors and activators of the overproduced proteins. Thus it is clear that the method can be generalized to a wide variety of genes encoding proteins which are involved in cellular growth control in numerous cell types. These studies are described in more detail below.

First I tested the capability of pMV7-based expression vectors (pMT7 is my preferred transfer vector) to produce several different types of proteins in various cell lines. I used the cDNA sequences encoding the following proteins: hypoxanthine/guanine phosphoribosyltransferase (HGPRT), the human T4 lymphocyte cell surface antigen, the human T8 lymphocyte cell surface antigen, and ornithine decarboxylase (ODC). In each case the pMV7 vector was capable of producing high levels of expression of the relevant gene thereby resulting in overproduction of the corresponding protein product.

Once I had verified that the pMV7-derived expression vector could reproducibly generate cell lines which stably overproduced proteins I then tested additional genes which encode proteins, other than PKC, which are also involved in cellular growth control.

In further experiments, I have tested the method using an activated c-H-ras oncogene (T24), again in analogous fashion to the techniques described herein, and again with analogous results to those described herein for the PKC cDNA clone. Thus, the Method can also be used for the rapid development of a p21 ras inhibitor. Taken together, the results described in this application demonstrate directly that the Method described herein is clearly generalizable to any gene which is involved in any way in cellular growth control.

In further studies, we have generated additional cell lines which stably overproduce other proteins-of-interest (POIs). In one set of studies, we have introduced the beta1 form of protein kinase C (PKC) into a bone-marrow derived cell line, FDC-P1, and demonstrated that the resulting cell lines are profoundly affected by PKC activators. In a second set of studies, we have developed a screening assay system for insulin agonists and antagonists which utilizes cell lines which stably overproduce the insulin receptor. In these additional studies we have utilized different cell lines, expression vector systems, cDNAs encoding other POIs, and in general varied all of the major parameters of our initial studies in order to demonstrate the true generalizability of the system to any type of cell and any protein-of-interest.

The preferred protein inhibitor/activator drug screening method of the present invention comprises the following steps:

1. Construction of an expression vector which is capable of expressing the protein of interest in the selected host by inserting a gene encoding that protein into a transfer vector. The gene may be inserted 3' of a promoter already borne by the transfer vector, or a gene and a promoter may be inserted sequentially or simultaneously.

2. Introduction of the expression vector (a) into cells which produce recombinant retrovirus particles, or (b)

directly into host cells which will be used for subsequent drug screening tests (the resulting cells are called herein "test" cells).

In parallel, the transfer vector (i.e., the vector lacking the gene of interest and possibly a linked promoter but otherwise identical to the expression vector is preferably also introduced into the host cells. Cell lines derived from this latter case will be used as negative controls in the subsequent drug screening tests. Alternatively, the unmodified host cells may be used as controls.

If 2a was employed, after an appropriate time (usually 48 hours), media containing recombinant virus particles is transferred onto host cells so as to obtain test or control cells.

3. The test and control cells are transferred to selective growth medium containing the appropriate drug which will only allow those cells harboring the expression vector containing the selectable marker gene (as well as the gene or cDNA of experimental interest) to grow. After an appropriate selection time (usually 7–10 days), individual clones of cells (derivative cell lines) are isolated and propagated separately.

4. Each independent cell line is tested for the level of production of the POI. By this method, a range of cell lines is generated which overproduce from a few fold to more that 100-fold levels of the POI. In parallel, the control cell lines which contain only the transfer vector alone (with the selectable marker gene) are also assayed for their endogeneous levels of the POI.

5. Each independent line is then tested for its growth capability in soft agar (or methocel. or any other similar matrix) of various percentages and containing different types of growth media until cell lines are identified which possess the desired growth characteristics as compared to the control cell lines.

6. Each cell line is also tested for its ability to form "foci", or areas of dense cellular growth. in tissue culture plates using media containing various percentages and types of serum (20%, 10%, 5% serum, fetal calf serum, calf serum, horse serum, etc.) and under various conditions of growth (e.g. addition of other hormones, growth factors, or other growth supplements to the medium, temperature and humidity variations, etc.). In these tests, the cells are maintained at post-confluence for extended periods of time (from two to eight weeks) with media changes every three days or as required. Such growth parameters are varied until cell lines are identified which possess the desired foci formation capacity relative to the control cell lines under the identical conditions.

7. After a cell line possessing the required growth characteristics is identified, the cells are grown under the conditions determined in (5) above with the growth medium supplemented with either crude or purified substances which may contain biologically active agents specific to the POI. Thus, crude or purified substances possessing the latter properties can be rapidly identified by their ability to differentially alter the growth properties of the experimental cells (which overproduce the POI) relative to the control cells (which do not). This can be done rapidly even by simple observation with the naked eye, since the colonies which grow in soft agar after 2 weeks are easily seen even without staining, although they may be stained for easier detection.

Similarly, if the post-confluence foci formation assay is chosen, the foci which result after approximately two weeks can be easily seen with the naked eye, or these foci can also be stained. For screening very large numbers of compounds (tens of thousands or more), the entire procedure can be performed on 96 well tissue culture plates. This applies equally well for either the soft agar growth assay or the tissue culture foci formation assay. Results of the assays can be rapidly determined by measuring the relative absorbance of the test cells as compared to the control cells (at 500 nm, or another appropriate wavelength). Absorbance readings may be rapidly performed in a 96-well plate absorbance reader such as the "Titer-tek" plate reader, or any of several analogous apparatus currently available. In this fashion, thousands of compounds could be screened per month for their biological activity with very low labor and materials costs.

Furthermore, if antigen expression varies on teh test cells expressing high levels of the POI relative to the control cells, a simple Enzyme-Linked Immunoadsorption Assay (ELISA) could be performed and an antibody specific to the antigen.

While the assay may be performed with one control cell line and one test cell line, it is possible to use additional lines, tests lines with differing POI levels. Also additional sets of control/test lines, originating from other hosts, may be tested.

Specific examples implementing the series of steps described above are as follows.

EXAMPLE 1

If one were interested in screening for a protein kinase C (PKC) inhibitor, cell lines would be generated and selected which grow well in soft agar (as a result of their overproduction of any form of PKC) and yet show an enhancement of their growth when compounds which are known to stimulate PKC are added to the growth medium. Appropriate control cells, of course, would not exhibit any of these characteristics. Screening for a potent PKC inhibitor could then be performed by searching for those substances which could selectively inhibit the soft agar (anchorage-independent) growth of the PKC-overproducing cell lines. Alternatively, since the PKC-overproducing cells also form small, dense foci in tissue culture, one could also screen for substances which inhibit this foci formation.

Described below are the detailed aspects of the relevant techniques and methods used to apply the principles of the invention to the problem of developing a system useful for screening for potent inhibitors of protein kinase C (PKC), a high-affinity intracellular receptor for tumor-promoting agents. The cell lines which resulted from the application of this method are highly sensitive and responsive both to agents which activate PKC as well as to those which inhibit PKC.

Construction of plasmid pMV7

The construction of pMV7 was begun with plasmid pPyori which contains the polyoma virus origin of replication cloned into the unique BamHI site of pML-1 (Lusky and Botchan, 1981). This plasmid replicates in murine cells that contain the polyoma T antigen (Dailey and Basilico, 1985). Plasmid pMV (Perkins et al., 1983) was then cleaved with HincII and BglI. The 2.29 kb fragment that contains the Molosey Leukemia Virus Long Terminal Repeats (LTR), the packaging site, the splice donor site, and the proline tRNA biading site was isolated.

During the original construction of pMV (Perkins et al., 1983) 3.95 kb was removed from the MSV genome by cleaving with PstI. This left a PstI site situated 380 bps 3' from the tRNA binding site and 308 bps from the 5' end of the 3' LTR (Reddy et al., 1981). Xho I linkers were added to

the HincII-BglI fragment, and to pPyori after it had been cleaved with EcoRI and HindIII. The two fragments were ligated, after activation of the linkers, and a plasmid, designated pMV-3, that contained the Moloney Virus control elements was isolated.

The unique EcoRI site was removed from this vector by digesting the plasmid with EcoRI and treating the linear molecules with T4 polymerase. These molecules were recircularized and a plasmid, pMV-4, lacking the EcoRI site, was isolated. An EcoRI linker was inserted into this plasmid at the PstI site between the Moloney LTRs; the resultant plasmid was designated pMV-5.

The dominant selectable marker (neo) was added to pMV-5. The first step was isolating a 1.9 kb BamHI-SalI fragment from pIPB1. This fragment contains the Herpes Simplex virus thymidine kinase (tk) promoter region and the coding sequence for the bacterial neogene (neomycin phosphotransferase). This fragment was blunt-ended with T4 polymerase, ClaI linkers were added and the fragment was cloned into the Cla I site 165 bps 3' to the EcoRI site in pMV-5, between the LTRs. This plasmid was designated pMV5-tk neo. The tk promoter has an EcoRI site 70 bps 5' to the start of transcription. This EcoRI site was removed by partially digesting pMV5-tkneo with EcoRI, isolating the linear full length cut species, filling in the ends with T4 polymerase and recircularizing the molecule. A plasmid was chosen in which the EcoRI site previously present in the tk promoter sequence was removed, but the EcoRI site 537 bps 3' to the start of transcription of the 5' LTR was retained. This plasmid was designated pMV7 and a map of this plasmid is shown in FIG. 1B.

This vector consists of the 5' and 3' Moloney Murine Leukemia Virus (MoMuLV) LTRs, the MoMuLV RNA packaging site 3' to the 5' LTR, an Eco RI cloning site, a modified Herpes Simplex Virus thymidine kinase (tk) promoter (lacking an Eco RI site at −79 bp), and the selectable marker gene neo. cDNA clones inserted into the EcoRI cloning site are under the transcriptional control of the 5' LTR, whereas the neogene is independently transcribed by the tk promoter. This structure favors maintenance of the functional integrity of the selectable marker without interfering with the expression of the 5' (unselected) cDNA sequence. It is known (Maddon et al., 1986; Daley et al., 1987) that when various cDNA sequences are inserted into the EcoR1 site of pMV7 they can be readily transferred into recipient cells by virus-mediated passage, are stably expressed, and yield high-level production of the corresponding protein.

Nucleotide Sequencing and Expression Vector Construction

Nucleotide sequencing of the PKC cDNA clone RP58, a full-length clone isolated from a rat brain cDNA library, which corresponds to the previously reported clone RP41, was performed as previously described (Housey et al., 1987). The full-length cDNA sequence of RP58, which encodes PKCbeta1 (FIG. 1A), was subcloned into the EcoRI site of plasmid pMV7 using standard methodology (Maniatis et al., 1983). The general structure of pMV7 is shown in FIG. 1B. The construct resulting from insertion of the PKCbeta1 gene is designated pMV7-PKCbeta1.

Isolation of cell lines stably overexpressing PKC

20 ug of CsCl banded pMV7 or pMV7-PKCbeta1 plasmid DNA were transfected (Graham and van der Eb, 1973; as modified by Wigler et al., 1977) onto subconfluent "Psi-2" cells (Mann et al., 1985). After 48 hrs the culture medium was collected, filtered through a 0.45 um filter and stored at −70° C. Recipient subconfluent Rat-6 fibroblasts (5×10^5 per 10 cm plate) were infected with the virus-containing medium in 2 ug/ml polybrene for 48 hrs. The cells, grown to confluence, were then trypsinized and replaced in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (CS) (Flow Laboratories) with 200 ug/ml of the neomycin derivative G418 (Geneticin). Resistant colonies were cloned by ring isolation after 1 week of G418 selection.

After approximately ten days of growth in selective medium, ten individual G418-resistant clones were isolated and maintained independently in G418-containing medium. These lines were designated R6-PKCbeta11 through R6-PKCbeta110 (abbreviated as R6-PKC1 through R6-PKC10). In parallel, a set of control Rat 6 lines was generated by transfection of the plasmid pMV7 (lacking the PKC cDNA insert) onto W-2 cells, infection of recipient Rat-6 cells, and selection for G418 resistance as described above for plasmid pMV7-PKCbeta1. Similarly, after ten days of growth in the G418-containing medium, five individual, well-isolated G418-resistant clones were then isolated and maintained independently. These control lines were designated R6-C1 through R6-C5.

RNA Isolation and Blot Hybridization Analyses

Poly A+ RNA isolations, gel electrophoresis, and blot hybridization analyses were performed as previously described (Housey, et. al., 1985). RNA molecular weight markers were obtained from Bethesda Research Laboratories. The 2.7 kb cDNA insert of RP58 (see above) was subcloned into plasmid pKS(+) (Stratagene Cloning Systems) to yield a plasmid designated pS2-RP58. A ^{32}P-labelled probe was prepared from pS2-RP58 and used under high-stringency hybridization conditions as previously described (Housey et al., 1987).

Purification and Assay of PKC Activity From Tissue Culture Cells

The total PKC activity (membrane-associated plus cytosolic) present in cultured cells was determined after partial purification of cellular extracts as follows.

Three 10 cm plates of confluent cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS) and then 10 ml of homogenization buffer (20 mM Tris, pH 7.5, 5 mM EGTA, 5mM EDTA, 15 mM 2-mercaptoethanol, 10 ug/ml soybean trypsin inhibitor, 10 ug/ml leupeptin, 40 ug/ml phenylmethylsulfonyl fluoride), containing 0.1% Triton X-100 were added. The cells were then scraped from each of the plates, pooled and disrupted with 25 strokes in a Dounce homogenizer. The homogenate was transferred to a 15 ml disposable polystyrene tube, centrifuged at 2000×g for 5 minutes at 4° C., and the supernatant was loaded onto a 1 ml DEAE Sephacel column previously equilibrated with 10 ml homogenization buffer, at 4° C. The column was washed with 10 ml homogenization buffer and then the bound enzyme was eluted with 3 ml of homogenization buffer containing 0.5M NaCl. Total protein concentrations were determined by the method of Bradford (1976).

The PKC activity present in the above-described partially purified cell extracts above was assayed immediately after isolation. The synthetic peptide R-K-R-T-L-R-R-L, corresponding to amino acids 651–658 of the epidermal growth factor receptor (Ullrich et al., 1984), was synthesized on an Applied Biosystems model 430A peptide synthesizer, purified by high-performance liquid chromatography, lyophilized, and stored at −20° C. The Threonine at position 654 is an in vivo substrate for PKC (Hunter, et al., 1984; Davis and Czech, 1985). This synthetic peptide is a highly specific substrate for PKC activity in vitro. (Watson et al., 1987; Woodgett, et al., 1986).

The purified material was then redissolved in sterile water at a final concentration of 100 uM and used as the' phos-

phoacceptor substrate in the PKC assays. The general method of assay has been published in detail elsewhere (O'Brian et al., 1985). In most cases, 100 uM synthetic peptide was substituted for 2 mg/ml histone III-S as the phosphoacceptor substrate.

Cell extracts were prepared 48 hours after the cells had reached confluence. As shown in Table 1(a), eight of the ten cell lines generated by infection with the pMV7-PKCbeta1 construct (lines R6-PKC1, R6-PKC3, and R6-PKC5 through R6-PKC10) contained marked increases in PKC activity when compared to the control lines (R6-C1, -C2, and -C3). It is remarkable that cell line R6-PKC3 contained a 53 fold higher level of PKC activity than that present in the control cells. Two of the lines (R6-PKC2 and R6-PKC4), however, did not display a significant increase in PKC activity, yet they presumably had integrated the pMV7-PKC construct as evidenced by their continued G418 resistance. Subsequent studies verified that these two cell lines contained deletions in the cDNA clone encoding PKCbeta1.

In additional studies I found that the very high PKC activity in extracts of R6-PKC3 seen in the presence of 1 mM Ca^{2+} and Phosphatidylserine was also apparent in the presence of 1 mM EGTA. 100 ng/ml TPA and phosphatidylserine. This very high activity was also seen when we employed histone III-S, rather than the above-described synthetic peptide, as the substrate for phosphorylation. Furthermore, even after 24 weeks of continuous growth and serial passage, the cell lines R6-PKC1 through R6-PKC6 displayed essentially the same levels of PKC activity shown in Table 1.

Autophosphorylation and Gel Electrophoresis of PKC

Cell extracts purified as described above were incubated under conditions which favor the autophosphorylation of PKC, as follows. One hundred ug of partially purified protein extract was incubated in a reaction mixture containing 80 ug/ml phosphatidylserine, 1 mM $CaCl_2$ (or 1 mM EGTA and 100 ng/ml TPA), 5 mM $MgCl_2$, and 30 uM ATP containing 100 uCi [gamma-^{32}P]ATP (New England Nuclear, NEG035). The purity of the radioactive ATP is critical to obtain reproducible autophosphorylation of PKC.

Under these conditions, it has been previously shown that PKC undergoes an autophosphorylation reaction which results in the phosphorylation of several sites on the intact enzyme (Walton et al., 1987; Huang et al., 1986, Woodgett and Hunter, 1986; Kikkawa, 1982).

Reactions were incubated at room temperature for 10 minutes and then stopped by the addition of SDS-PAGE loading buffer containing 2-mercaptoethanol. This material was then subjected to discontinuous SDS-PAGE by a modification of the method of Laemmli (1970). Twenty ug of total protein were loaded onto each lane. Following electrophoresis, the gels were fixed in 50% acetic acid, 10% ethanol, dried, and autoradiographed on Kodak XAR-5 film.

Autoradiographs of these gels (FIG. 2) revealed that the extracts prepared from four cell lines that had high PKC activity (R6-PKC1, -PKC3, -PKC5, and -PKC6, see Table 1(a)) displayed a prominent phosphorylated protein band which was about 75 kd in size, corresponding to the size of an autophosphorylated preparation of PKC obtained from rat brain (Huang et al., 1986; Housey et al., 1987). When examined in an immunoblot assay this 75 kd band also reacted with an antibody to the beta1 form of PKC (Jaken and Kiley, 1987). The control cell lines R6-C1, -C2 and -C3, and the cell lines R6-PKC2 and R6-PKC4, which did not have increased levels of PKC (see Table 1(a)) did not show this 75 kd phosphorylated band (FIG. 2), nor did they contain any bands which reacted with the antibody to the beta1 form of PKC. It is of interest that the samples obtained from the four cell lines that produced high levels of PKC also displayed weaker but distinct phosphorylated protein bands that were about 73, 60, and 49 kd in size, which were not seen (or only faintly detected) in the extracts from cells that did not have increased levels of PKC (FIG. 2). These bands may represent degradation fragments of the 75 kd PKC molecules, or specific cellular proteins that are phosphorylated by PKC.

The above-described phosphorylated protein bands were seen when either 1 mM Ca2+ plus phosphatidylserine or 100 ng/ml TPA plus phosphatidylserine were used as cofactors for PKC activation (compare even and odd numbered lanes in FIG. 2). When, however, extracts from cell lines producing high levels of PKC were incubated in an autophosphorylation reaction in the absence of such cofactors, the 75 kd band and the additional smaller bands described above were not detected. These results, taken together with the negative results obtained with extracts from the control cells (FIG. 2), clearly indicate that the phosphorylated bands reflect PKC activity.

Phorbol Ester (^{3}H-PDBU) Binding Assays

Since it has been shown that PKC is a high affinity intracellular receptor for the phorbol ester tumor promoters (for review, see Nishizuka, 1986). I also assayed a subset of the cell lines for 3H-PDBu binding using a previously described intact cell assay (Horowitz et al., 1981).

Cells were plated at 1×10^5 per 4 cm well on day 1, the medium was changed on day 2 and the cells then assayed on day 3. The monolayer was washed with 4 ml of DMEM (without serum), then 2 ml of DMEM containing 50 nM ^{3}H-PDBU (New England Nuclear; 8.3 Ci/mmol) was added and the plates were incubated for 1 hr at 37° C. to determine total binding. The fraction of the total binding that represented specific binding was determined by the addition of a 1000-fold excess of unlabelled PDBU (LC Services) to the 3H-PDBU stock (Horowitz et al., 1981). The plates were washed 3× with 4 ml ice-cold PBS. The cells were solubilized in 1 ml 1% SDS/10 mM DTT for 2 hrs at 37° C. The lysate was transferred to a scintillation vial and counted. Replica plates were used to determine the number of cells per plate and the specific binding data expressed as nanomoles 3H-PDBu bound/10^6 cells. Scatchard analyses were performed as previously described (Horowitz et al., 1981).

I found that the R6-PKC cell lines 1,3,5 and 6, all of which had high PKC enzyme activity, also had a marked increase in 3H-PDBu binding, whereas the cell line R6-PKC4, which did not show an increase in 3H-PDBu binding when compared to the two control cell lines R6-C1 and R6-C2 (Table 1(a)). Scatchard analyses of the control cell line R6-C1 and of the R6-PKC3 cell line, performed as previously described (Horowitz et al., 1981), indicated that the number of high affinity receptors in the two cell lines was 1.6×10^5 and 1.4×10^6, respectively. The affinity constants were approximately the same in both cell lines (Kd=16 nM). Thus, under the assay conditions used, the R6-PKC3 cells contain about ten times the level of high affinity phorbol-ester binding sites as the control cells. It is apparent that the cell lines that express very high levels of PKC also have a significant increase in phorbol ester binding sites.

Assays for PKC-related RNA transcripts

In view of the above results, it was of interest to analyze the poly A+ RNA fraction of several of the cell lines described in Table 1(a) for the size and abundance of RNA transcripts containing sequences homologous to PKCbeta1.

Poly A+ RNA was separated on 1% agarose, 6%-formaldehyde gels, blotted onto nylon membranes,

hybridized to a 32P-labelled DNA probe prepared from the full-length PKCbeta1 cDNA, and autoradiographed, as previously described (Housey, et al., 1986). As shown in FIG. 3, the lines that contained elevated levels of PKC activity (R6-PKC1,-PKC3,-PKC5, and-PKC6, see Table 1(a)) contained a prominent 6.6 kb RNA species which corresponds to the predicted size for a mRNA transcript that initiates in the 5 ' LTR and terminates in the 3 ' LTR of the pMV7-PKCbeta1 construct. This transcript was most abundant in the R6-PKC3 cell line (FIG. 3) which also expresses the highest level of PKC activity (Table 1).

On the other hand, lines R6-PKC2 and R6-PKC4, which showed no significant elevation of PKC activity (Table 1(a)), produced truncated mRNA's of approximately 5 kb and 4.8 kb, respectively. The abundance of the latter transcripts was much lower than that of the 6.6 kb transcripts present in the cell lines that expressed high levels of PKC. The neo+ phenotype and the lack of PKC activity in cell lines R6-PKC2 and R6-PKC4 suggest that the truncated mRNAs reflect deletions in PKC coding sequences in the integrated constructs carried by these cell lines. Indeed, genomic DNA blot hybridizations of clones R6-PKC2 and R6-PKC4 indicated that they contained deletions in the PKCbeta1 cDNA sequence. Neither in the parental Rat 6 cell line, nor in any of the experimental cell lines derived from these cells, was it possible to detect evidence of an endogenous transcript homologous to the PKCbeta1 probe (FIG. 3). Thus, in these cells, there is negligible expression of the endogenous gene encoding PKCbeta1.

Assays of Growth in Monolayer Culture and in Soft Agar

Cells were seeded at a density of 10^4/plate in a series of 60 mm plates, in 5 ml DMEM plus 10% CS. Twenty-four hours later, cells in triplicate plates were trypsinized and counted. This point was designated "day 0." The remaining cultures were then grown in the respective medium (i.e. plus or minus 100 ng/ml TPA) with fresh medium changes twice per week. Cell counts per plate were then determined on triplicate plates during the remainder of the growth curve (FIG. 3). The results obtained were analyzed for exponential doubling time and saturation density (Table 2). To assess growth in soft agar (anchorage independence), 2×10^4 cells were suspended in 2 ml of 0.3% Bacto agar (Difco Laboratories, Detroit, Mich.) in DMEM containing 20% fetal calf serum (FCS) and overlayed above a layer of 5 ml of 0.5% agar in the same medium, on 60 mm petri dishes. The cells were then overlaid with DMEM plus 20% FCS every 4 days. At the end of 30 days, colonies were stained with the vital stain 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolinium chloride hydrate (INT) (Sigma Chemical Co., St. Louis, Mo.) for 48 hours at 37° C., in an incubator with 5% CO2 (Schaeffer and Friend, 1976), and the number of colonies counted under low power on an inverted phase microscope. The data are expressed as "cloning efficiency", i.e. number of colonies greater than 0.05 mm per plate ×100 divided by the number of cells originally seeded per plate.

Screening of a Known Activator of PKC

We found that cell lines which overproduce PKC display an exaggerated morphologic response to 12-O-tetradecanoyl phorbol-13-acetate (TPA) and altered growth control.

Changes in Morphology

To further characterize the phenotypic changes which occurred in the cell lines that overproduce PKCbeta1, lines R6-PKC3 and R6-PKC5, which contain 53- and 20-fold elevations of PKC activity, respectively, were first examined in detail with respect to their morphology, in comparison to the control cell line R6-C1. As shown in FIG. 4, Panel A, in the absence of TPA treatment all three cell lines showed the characteristic fusiform morphology of monolayer cultures of the normal parental Rat 6 fibroblast cell line. At 24 hours after treatment with 100 ng/ml TPA (FIG. 4, R6-C1: panel D), the control cell line displayed more elongated and dendritic cells and a criss-cross pattern, changes previously seen shortly after rodent fibroblasts are treated with TPA (Boreiko et al., 1980). These changes were, however, much more dramatic when the R6-PKC3 and R6-PKC5 cells were treated with TPA (R6-PKC3 and R6-PKC5, Panel D). This was particularly striking with the R6-PKC3 cells (which express the highest level of PKC) since they displayed very long cytoplasmic processes and numerous refractile cell bodies.

By 48 hours following exposure to TPA the morphology of the control cell line R6-C1 had returned to its normal appearance (FIG. 4, Panel E). On the other hand, the R6-PKC3 cells, and to a lesser extent the R6-PKC5 cells, continued to display an altered morphology. All of the cell lines were then exposed to a second, fresh dose of TPA (100 ng/ml) and examined 24 hours later (FIG. 4, Panel F), i.e., 72 hours after the first dose of TPA. The control cells failed to respond, in terms of morphologic change, to the second dose of TPA whereas the R6-PKC3 cells continued to display their altered morphology as well as an increase in cell density. The R6-PKC5 cells displayed only slight changes in morphology in response to the second dose of TPA.

Presumably, the very high level of constitutive production of PKC in the R6-PKC3 cell line is responsible for their exaggerated morphologic response to TPA as well as the failure of these cells to display the usual refractory response to TPA following an initial exposure. In normal cells, the latter response appears to be due to "down-regulation" of endogenous PKC activity. Since the R6-PKC5 cells have an intermediate level of PKC, it is not surprising that their morphologic responses to TPA are intermediate between those of the control cells and the R6-PKC3 cells.

Growth Curves

It was also of interest to determine the growth rates of these cells in monolayer culture. Detailed growth curves were performed on R6-C1, R6-PKC3 and R6-PKC5 cells in 10% calf serum and DMEM medium, in the absence and presence of 100 ng/ml TPA. The data obtained are summarized quantitatively in Table 2.

In the absence of TPA, the R6-C1 control cell line displayed the longest doubling time (26.4 hours) and the lowest saturation density (3.4×10^6 cells/plate); the R6-5 cell line had a shorter doubling time (24.9 hours) and a higher saturation density (4.8×10^6 cells/plate); and the R6-PKC3 cell line had the shortest doubling time (24.2 hours) and the highest saturation density (5.7×10^6 cells/plate). The presence of TPA decreased the doubling times, and also increased the saturation densities of all three cell lines, but the enhancement by TPA was particularly striking in the case of R6-PKC3 (Table 2).

When the cell lines were maintained in the presence of TPA for a longer period of time, the R6-PKC3 cells, but not the R6PKC5 or R6-C1 cells, showed a decline in cell density. The latter effect was due to the fact that when the R6-PKC3 cells were maintained at high cell density in the presence of TPA they became less adhesive and tended to detach from the plate.

Thus, even in the absence of TPA the R6-PKC3 cells, (which have the highest level of PKC), and to a lesser extent the R6-PKC5 cells (which have an intermediate level of PKC), exhibit an enhancement of their growth properties

which is even greater than that seen when the R6-C1 control cells are grown in the presence of TPA. Moreover, in the presence of TPA these differences in growth properties between the control and R6-PKC3 cells are even more striking.

Foci Formation

In additional studies, monolayer cultures were maintained at post-confluence for an extended period of time (28 days), with media changes every 3 days, in the absence of TPA. Whereas the control R6-C2 cell line remained a fairly uniform monolayer, after about 21 days the R6-PKC3 cell line developed numerous dense foci which were approximately 0.1–0.3 mm in diameter (FIG. 5). Furthermore, the R6-PKC3 cultures displayed numerous cells with a highly vacuolated cytoplasm which were scattered throughout the monolayer, but were not seen in the R6-C2 control culture. When the dense foci seen in the R6-PKC3 culture were picked and further passaged they grew like the parental R6-PKC3 cells and did not display a morphology typical of malignantly transformed cells. It may be surmised that these dense foci, and the vacuolated cells, reflect physiologic rather than genetic changes induced by the high level of PKC activity.

Growth on Soft Agar

I also assayed these cell lines for their ability to form colonies in soft agar, since with rodent cells the acquisition of anchorage-independent growth often correlates with tumorigenicity (Freedman and Shin, 1974). As shown in FIG. 6, when 2×10^4 cells were plated in 0.3% soft agar, both the R6-PKC3 and the R6-PKC5 cells formed numerous small colonies, whereas the control R6-C1 cells (and the parental Rat 6 cell line) failed to grow and persisted as single cells. In addition, when TPA (100 ng/ml) was added to the agar medium, the colony sizes and cloning efficiencies of the R6-PKC3 and RC-PKC5 cells were enhanced (FIG. 6 and Table 2), but the R6-C1 cells still failed to grow in agar.

The cloning efficiencies and colony sizes of the R6-PKC3 cultures were always greater than those of the R6-PKC5 cultures, both in the absence and presence of TPA (Table 2), presumably reflecting the higher level of PKC activity in the former cell line. Thus, it is clear that the overproduction of PKC is associated with the acquisition of anchorage-independent growth in Rat 6 cells. The sizes of the colonies formed in agar by the PKC cell lines are smaller than those formed by Rat 6 cells transformed by an activated c-H-ras oncogene, which have a diameter of about 0.5–1.0 mm.

Further Screening of Activators and Inhibitors of PKC

Additional compounds tested included the tumor promoters teleocidin, aplysiatoxin, and mezerein, all of which are known activators of PKC (O'Brian, et al., CSH. 1985). Furthermore, a known inhibitor of PKC, H-7 (Kawamoto and Hidaka, 1984), also modulated the growth of the cells in the expected manner.

Moreover, this method has been used to establish that the anti-estrogen tamoxifen (O'Brian, et al, Cancer Res., 1985), which inhibits PKC enzyme activity in a cell-free assay, is capable of completely inhibiting the growth in agar of all of the cell lines overproducing PKC. Inhibition of the growth of the R6-PKC3 cells in agar in the presence of tamoxifen provided critical evidence that tamoxifen could inhibit PKC-mediated stimulation of cellular growth. See the data of Table 3.

Furthermore, the concentration of the inhibitor necessary to completely inhibit the growth of each cell line was roughly proportional to the amount of PKC being overproduced in that particular cell line. In other words, there is a direct relationship between the molar amount of inhibitor required to prevent cell growth and the molar amount of PKC present in each cell line.

In addition, I have demonstrated the analogous relationship between the molar amounts of PKC activators and the molar amounts of PKC present in cells. In other words, there is a direct relationship between the molar amount of activator required to stimulate cell growth and the molar amount of PKC present in cells. Thus, this work establishes, for the first time, the fact that stable overproduction of a protein in mammalian cells can result in a novel cellular phenotype(s) (in this case anchorage independence) which can be directly modulated by chemical agents which interact with the protein.

EXAMPLE 2

If one were interested in screening for a potent inhibitor of the c-H-ras oncogene product (the p21 protein) then one would generate cells which grow well in soft agar with appropriate media conditions when p21 is stably overproduced at a certain level, but not at all when p21 is present at wild type levels. Screening for a potent p21 inhibitor could then be performed as described in Example 1 above.

EXAMPLE 3

The same basic techniques would also apply to genes (or cDNA sequences) which have been mutated either by laboratory design (e.g. site-directed mutagenesis) or as a result of naturally occurring events. Thus, any of the known point mutations in the ras oncogene which result in greater capability by the mutated gene to transform normal cells to cancerous ones could be employed in the same basic procedures as described above.

EXAMPLE 4

The previously mentioned retroviral expression vector pMV7 was utilized to introduce a cDNA encoding the beta1 isoform of PKC into FDC-P1 cells. FDC-P1 is a bone marrow-derived cell line which requires interleukin-3 (IL-3) for growth. In these studies, the hemopoietic cell line FDC-P1 was utilized in order to demonstrate the utility of the screening method even in cells which have very stringent growth requirements and would otherwise be very difficult to maintain in culture.

The following abbreviations are used herein: SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis (beta aminoethyl ether) N,N,N',N'-tetraacetic acid; EDTA, ethylene diamine tetraacetic acid; TPA, 12-O-tetradecanoyl phorbol-13-acetate (also known as PMA); CHO, Chinese hamster ovary cells; DEAE, diethyl aminoethyl.

Cell Culture

FDC-P1 cells were routinely maintained in Dulbecco's modified eagle's medium containing 10% iron-supplemented calf serum. We added 10% WEHI-3B cell-contained medium as a source of IL-3 and subcultured the cells twice weekly to give 2×10E5 cells/ml.

Isolation of cell lines stably overexpressing PKC

Subconfluent [PSI]-2 cells expressing a cDNA clone encoding the complete PKCbeta1 sequence (Housey et al., 1988) and containing the G418 resistance gene were irradiated with 3000 rad. These cells were cocultivated with FDC-P1 grown as described above, and 8 ug/ml of polybrene was added. After 48 hours, the FDC-P1 cells were pelleted, plated in 35 mm petri dishes containing 0.3% agar, 10% calf serum, and 10% WEHI conditioned medium, and selected with 0.3 mg/ml of G418. After 10 days colonies were aspirated from the agar and grown in liquid medium.

RNA isolation and blot hybridization

Total RNA was extracted (Chomczynski, 1987) from FDC-P1 cells and run on a 1.5% agarose gel, blotted to nitrocellulose, and probed with the nick-translated EcoR1-Pst1 fragment of the RP-58 cDNA encoding PKCbeta1 (Housey, 1988). In separate experiments nitrocellulose blots were probed with either the BamH1 fragment of the murine GM-CSF cDNA (Lee, 1985) or the XhoI fragment of the murine IL-3 gene (Yokota, 1984), clones donated by Dr. Lee and labelled by nick-translation to 1×10E8 cpm/ug. The labelled PstI fragment of tubulin was used to examine RNA loading.

Western blotting of PKC

FDC-P1 cells were placed directly into a buffer containing 1% SDS; 2 mM EGTA; 2 mM EDTA; 20 mM Tris-HCl, pH 8.0, and 1% beta mercaptoethanol. The sample was boiled and pelleted for 30 min. at 20 degrees C. at 200,000×g. Then 80 ug of supernatant protein was electrophoresed on a 10% SDS polyacrylamide gel, and the proteins were transferred to nitrocellulose. The blot was blocked with bovine serum albumen and was probed with an antibody made to a conserved region of PKC (LLNQEEGEYYNVPIPE) which recognizes all forms of the enzyme (Stabel, 1987). The PKC standard was purified from rat brain by previously published procedures (Kraft, 1988).

Stable Overproduction of PKCbeta1 in FDC-P1 Cells

In order to generate FDC-P1 cells which stably overproduced PKCbeta1, we cocultured FDC-P1 cells, which are dependent upon a source of IL-3 for continued growth, with [PSI]-2 cells which produce a retrovirus encoding the complete PKC beta1 cDNA clone in the pMV7 expression vector. After 48 hours of cocultivation, the FDC-P1 cells were plated in soft agar in medium containing G418 and IL-3, and after 10 days of selection several colonies were aspirated and grown in liquid culture.

Approximately 30 of these clones were screened by western blotting with a PKC antibody which recognizes all forms of PKC. A number of clones demonstrated elevated levels of PKC beta1 protein by Western blot analysis. Northern blot analyses were consistent with the western blot results and demonstrated that clones 40, 43, and 51 exhibited hybridizable bands to the EcoR1-PstI fragment of the PKC-beta1 clone, whereas the parental cell line and clone 2 (which lacked any PKCbeta 1-specific mRNA), were negative. Furthermore, the total PKC activity in these cells was examined using histone IIIs as a phosphotransferase acceptor (Table 4). As expected, Clone 2 and the parental cell line had approximately the same PKC activity, whereas clones 40, 43, and 51 demonstrated approximately 20-fold increases in activity.

PKC Activators Induce Significant Morphologic Changes in FDC-P1 Cells That Stably Overproduce PKC

We then tested the morphologic responsiveness of the FDC-P1 cells to activators of PKC induce profound morphologic changes in the FDC-P1 cells. Several cell lines were plated at 3×10E4 cells/ml and treated with 100 nM TPA. After 12 hours, representative fields were photographed. The PKC beta1 overproducing cells exhibited profound morphologic changes, including a generalized rounding up of the cells followed by the rapid formation of dense, cellular clumping. The latter clumps are easily visualized with standard cell stains such as Giemsa, and stain much darker than the corresponding control cells under these conditions.

Screening Assay to Detect Activators and Inhibitors of PKC

Thus, as with our previous findings for other cell lines such as NIH 3T3, Rat 6, and C3H 10T 1/2, this system also provides a rapid, powerful cell-based assay for the discovery and characterization of both activators and inhibitors of PKC as follows. Compounds could be rapidly screened for new activators of PKC by testing their ability to induce cellular clumping in the Test cells, while leaving the Control cells essentially unaffected. In addition, if one wanted to screen for PKC inhibitors, compounds could be rapidly screened for their ability to inhibit the effects of PKC by testing their ability to prevent cellular clumping induced by TPA in the Test cells, while again leaving the Control cells essentially unaffected. In this latter test, Control and Test cells would be incubated simultaneously with 100 nM TPA and the individual compounds to be tested. After 12 hours the Control and Test cells would be stained and scored for the inhibition of cellular clumping in the Test cells while leaving the Control cells unaffected.

EXAMPLE 5

In further studies designed to test the utility of this system with other proteins of interest (POI), we have utilized the human insulin receptor as a model POI. Cell lines which stably overproduce the insulin receptor were used to develop an assay system which is capable of detecting both agonists and antagonists of insulin action.

In the majority of vertebrates, insulin is the primary hormone involved in the homeostatic control of blood glucose levels. The many effects of insulin action on cells include stimulation of glucose, protein, and lipid metabolism as well as effects on RNA and DNA synthesis (Metcalf, 1985). These actions of insulin on cells begin at the molecular level through the binding of insulin to the insulin receptor. The insulin receptor is present on virtually all mammalian cells, but its number varies from as few as 40 to more than 200,000 receptors per cell (Kahn and White, 1988, and references therein). The insulin receptor is a heterotetrameric glycoprotein consistent of two alpha and two beta subunits. The alpha subunits, Mr=135,000, are located entirely extracellularly, whereas the beta subunits are transmembrane proteins which possess intrinsic tyrosine kinase activity. The alpha and beta subunits are attached by disulfide bonds on the extracellular side of the cell membrane. Both the alpha and the beta chains of the mature receptor molecule are derived from a single chain precursor polypeptide (the "proreceptor") which in turn is encoded by a single gene.

It is now known that the beta subunit of the insulin receptor is a protein tyrosine kinase which phosphorylates substrate proteins on tyrosine residues. This tyrosine kinase activity of the beta subunit is stimulated by the binding of insulin to the alpha subunit, and it has been shown that the tyrosine kinase activity of the beta subunit is essential for insulin action on target cells (Kahn and White, 1988, and references therein).

Generation of CHO Cell Lines Stably Overproducing the Human Insulin Receptor

In additional demonstrations of the utility of the method, we have employed Chinese Hamster Ovary (CHO) cell lines which stably overproduce the human insulin receptor (HIR) (White et al., 1988). These cell lines were generated using approaches analogous to those previously described for the PKC-overproducing cell lines. However, in these HIR studies we utilized a different expression vector, distinct cell lines, and an alternative method of gene transfer (direct transfection as opposed to virus-mediated transduction as used in the PKC experiments previously described). Briefly, CHO cells were co-transfected with the transfer vector pSVEneo (containing the neomycin resistance gene) and the

expression vector pCVSVHIRc as previously described (White et al., 1988). After twenty four hours, the transfected cells were placed into 800 ug/ml G418 to select resistant cells. Approximately two weeks later, G418-resistant clones were isolated and subcloned. Clonal cell lines which express high levels of surface insulin receptors were selected by fluorescence-activated cell sorting. One such line (CHO/HIRC), which stably overproduced approximately 80,000 insulin receptors per CHO cell, was utilized in these experiments. A control CHO cell line (CHO/NEO), harboring only the pSVEneo transfer vector, were generated in analogous fashion. Detailed preliminary characterizations of this cell line have been described (White et al., 1988).

Morphologic and Growth Control Effects of Insulin Treatment on CHO Cells Stably Overproducing the Human Insulin Receptor

We then tested the ability of the CHO/HIRC and CHO/NEO cells to respond to the following insulin treatment protocol. Control cells, hereinafter termed CHO/NEO (which harbor pSVEneo alone), and the Test cells, termed CHO/HIRC (which stably overproduced about 80,000 insulin receptors per cell), were incubated in the presence and absence of insulin. Control (CHO/NEO) and Test (CHO/HIRC) cell lines were exposed to 400 nM of insulin and the resultant morphologic changes were observed and photographed using a Bausch and Lomb BL-200 phase-contrast microscope with a Nikon FM-200 camera attached.

After 18 hours the test cells began to exhibit profound morphologic changes, including increased refractility, cellular rounding, and decreased adherence to the culture plate (anchorage dependence). These effects were further pronounced at 24 hours after exposure. Indeed, by 48 hours after treatment, virtually all of the test cells had completely detached from the plate, whereas the control cells were still largely intact. If the test cells were transferred to new plates in medium lacking high levels of insulin, however, they were completely viable and would attach to the plate and continue to grow normally. Thus, although insulin treatment could rapidly induce the anchorage independent phenotype, the cells remained completely viable for future growth if insulin was subsequently removed from their growth medium.

Screening Assay to Detect Activators and Inhibitors of the Insulin Receptor

Thus, in yet another demonstration of the utility of our method, the system described above provides a rapid and powerful screening method for the discovery and characterization of insulin agonists and antagonists as follows.

Compounds may be screened by testing their ability to preferentially induce anchorage-independent growth in the Test cells relative to the Control cells. Each compound would be applied to both the Control and the Test cells growing in multi-well plates, and 48 hours later the wells would be stained with a simple dye such as Giemsa (Housey et al., 1988 and references therein).

Whereas the Control CHO/NEO cells would consistently remain attached to the plate and stain strongly with Giemsa, the Test CHO/HIRC cells would be induced to detach from the plate by compounds which mimic the effects of insulin and therefore would no longer be stained by Giemsa. A standard microtitre well plate-reader (such as those produced by SLT America, Inc.) could easily read the absorbance of each well due to the staining procedure(s) used.

Thus, in this manner thousands of compounds could be screened for their ability to mimic the effects of insulin. Such agents are, of course, insulin agonists. However, as with the methods described above for PKC, the method could also be utilized for the discovery of insulin antagonists by simply treating the Control and Test Cells with potential antagonist compounds in the presence of insulin.

By this latter approach, compounds capable of inhibiting the effects of insulin action would prevent the induction of anchorage independence in the CHO/HIRC cells, whereas compounds with no effect would be unable to prevent the induction of anchorage-independent growth in the Test Cells. Most importantly, this insulin receptor assay system demonstrates yet another direct application of the utility of stable overproduction of a protein-of-interest (POI) for the development of powerful assay systems capable of detecting activators or inhibitors of any POI.

Julius, et al., Science, 244: 1057–62 (Jun. 2, 1989) reports the production of a cell line which overproduces the serotonin receptor 5HTlc by cloning its cDNA into the expression vector pMV7. The transformed cell lines exhibited a change in morphology, specifically, the formation of foci. Mesulergine, a serotonin antagonist, completely blocked the formation of foci. This paper was published after the filing date of the parent application, Feb. 10, 1988, and is therefore cited merely to further demonstrate the broad applicability of the screening method described herein.

Further Modifications

Use of any expression vector capable of stably overexpressing a given gene in a recipient cell could be used with success in the procedures described herein. The retroviral vector which I used here was particularly well suited to the problem since I had designed it specifically for these purposes. However, other similar vector systems would work. Also, one could do co-transfection of an experimental gene inserted in one plasmid vector along with a second plasmid containing the selectable marker gene (rather than having both the experimental gene and the selectable marker gene on the same plasmid vector). This is more difficult and less efficient than using the pMV7 vector, but it would work to some extent.

Any growth medium, in addition to soft agar or methocel, which tends to prohibit the growth of normal, non-transformed cells, could also be used.

A culture of *E. coli* DH1 bearing the plasmid denoted pMV7-RP58 (pMV7-PKC beta1), was deposited under the Budapest Treaty with the American Type Culture Collection on Feb. 11, 1988, ATCC No. 67654. The deposit of this plasmid is not to be construed as an admission that the deposit is required for enablement or that the disclosure is limited to the deposited vector or gene.

TABLE 1(a)

PKC Activity and Phorbol Ester Binding in Rat 6 Cells Infected with pMV7 or pMV7-PKCbeta1 Constructs

	PKC Activity		3H-PDBU
Cell Line	Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control	Binding (pmol/10^6 cells)
R6-C1	100	—	1.6
R6-C2	85	—	1.3
R6-C3	150	—	1.5
mean ± s.d.	100 ± 34	1	
R6-PKC1	3480	23	12.7
R6-PKC2	85	1	ND
R6-PKC3	5840	53	9.9
R6-PKC4	190	2	0.7
R6-PKC5	2200	20	5.8
R6-PKC6	4600	42	7.1
R6-PKC7	2150	20	ND
R6-PKC8	3280	30	ND

TABLE 1(a)-continued

PKC Activity and Phorbol Ester Binding in Rat 6 Cells Infected with pMV7 or pMV7-PKCbetaI Constructs

Cell Line	PKC Activity: Specific Activity (pmol/min/mg prot)	PKC Activity: Fold Increase Relative to Control	3H-PDBU Binding ($pmol/10^6$ cells)
R6-PKC9	4990	45	ND
R6-PKC10	5050	46	ND

TABLE 1(b)

PKC Activity in NIH-3T3 Cell Lines Infected with pMV7 or pMV7-PKCbetaI Constructs

	PKC Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control
NIH-3T3 Cell lines (Controls)		
3T3-C1	110	—
3T3-C2	150	—
3T3-C3	90	—
mean ± s.d. (control lines)	115 ± 30	
NIH-3T3 PKC-Overproducing Cell Lines		
3T3-PKC1	2570	22
3T3-PKC2	3640	32
3T3-PKC3	1960	17
3T3-PKC4	1340	11
3T3-PKC5	4150	36
3T3-PKC6	2110	18
3T3-PKC7	5050	44

TABLE 1(c)

PKC Activity in C3H-10T1/2 Cell Lines Infected with pMV7 or pMV7-PKCbetaI Constructs

	PKC Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control
C3H-10T1/2 Cell lines (Controls)		
C3H-C1	115	—
C3H-C2	155	—
C3H-C3	130	—
C3H-C4	185	—
mean ± s.d.	145 ± 30	
C3H-10T1/2 PKC-Overproducing Cell Lines		
C3H-PKC1	2340	16
C3H-PKC2	5010	35
C3H-PKC3	950	7
C3H-PKC4	1360	9

TABLE 1(c)-continued

PKC Activity in C3H-10T1/2 Cell Lines Infected with pMV7 or pMV7-PKCbetaI Constructs

	PKC Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control
C3H-PKC5	4340	30
C3H-PKC6	7460	51

Legend to Table 1

Control cell lines were obtained by infecting rat-6-fibroblast, NIH-3T3 and C3H-10T1/2 cells with the pMV7 vector itself (lacking the PKC cDNA insert) whereas the PKC-overproducing cell lines were obtained from rat-6-fibroblast, NIH-3T3 and C3H-10T1/2 cells infected with the pMV7-PKCbeta construct, as described above. Total PKC activity was partially purified from each of the cell lines and assayed in the presence of 1 mM Ca2+ and 80 ug/ml phosphatidylserine, using the synthetic peptide R—K—R—T—L—R—R—L as substrate. Specific activity is reported as the amount of incorporation of 32P into the synthetic peptide substrate per milligram of protein per minute. All assays were done in duplicate and varied by less than 10%.

TABLE 2

GROWTH PROPERTIES OF RAT 6 CELL LINES OVERPRODUCING PKC AND THEIR RESPONSES TO TPA TREATMENT

		Growth in Culture: Monolayer		Growth in Culture: Agar	
Cell Line	TPA Add	Doubling Time (hrs)	Saturat. Density ($\times 10^4$)	Effic. (%)	Colony Size (mm)
R6C1	–	26.4	3.4	0	—
(control)	+	24.6	4.2	0	—
R6-PKC3	–	24.2	5.7	25.1	0.10–0.15
(test)	+	21.5	10.0	29.7	0.15–0.35
R6-PKC5	–	24.9	4.8	17.3	0.05–0.10
(test)	+	22.9	7.0	34.7	0.10–0.15

Table 2.

The cells were grown as described above. The "doubling times" relate to the initial exponential phase of cell growth and the "saturation density" represents the number of cells per 6 cm plate on day 11. The data are taken from the experiment shown in FIG. 3.

TABLE 3

Inhibition of Growth in Agar of PKC-Overproducing Cell Lines Using Various Inhibitors

Inhibitor	Cell Line	Conc. Inhibitor (uM)	Effic. of Growth In Agar (%)
H-7	R6-PKC3	0	27
H-7	R6-PKC3	2	25
H-7	R6-PKC3	5	22
H-7	R6-PKC3	10	17
H-7	R6-PKC3	50	4
H-7	R6-PKC3	100	0
H-7	R6-PKC5	0	20
H-7	R6-PKC5	2	21
H-7	R6-PKC5	5	16
H-7	R6-PKC5	10	4
H-7	R6-PKC5	50	1
H-7	R6-PKC5	100	0
Tamoxifen	R6-PKC3	0	25
Tamoxifen	R6-PKC3	5	27
Tamoxifen	R6-PKC3	20	22
Tamoxifen	R6-PKC3	50	15
Tamoxifen	R6-PKC3	100	6
Tamoxifen	R6-PKC3	200	0
Tamoxifen	R6-PKC5	0	19

TABLE 3-continued

Inhibition of Growth in Agar of PKC-Overproducing Cell Lines Using Various Inhibitors

Inhibitor	Cell Line	Conc. Inhibitor (uM)	Effic. of Growth In Agar (%)
Tamoxifen	R6-PKC5	5	19
Tamoxifen	R6-PKC5	20	17
Tamoxifen	R6-PKC5	50	8
Tamoxifen	R6-PKC5	100	0
Tamoxifen	R6-PKC5	200	0
Staurosporine	R6-PKC3	0	29
Staurosporine	R6-PKC3	0.001	29
Staurosporine	R6-PKC3	0.005	26
Staurosporine	R6-PKC3	0.010	15
Staurosporine	R6-PKC3	0.050	7
Staurosporine	R6-PKC3	0.250	0
Staurosporine	R6-PKC5	0	22
Staurosporine	R6-PKC5	0.001	21
Staurosporine	R6-PKC5	0.005	17
Staurosporine	R6-PKC5	0.010	10
Staurosporine	R6-PKC5	0.050	2
Staurosporine	R6-PKC5	0.250	0

TABLE 4

PKC Activity in FDC-P1 Cells Transfected with a Retrovirus Encoding PKCbeta1

Cell Line	PKC Activity (pmol/min/ug prot)	Fold Increase Relative to Parental Cell Line
Parental Control	78	1
FDC-P1 clone 2	64	1
FDC-P1 clone 4	1873	24
FDC-P1 clone 43	1558	20
FDC-P1 clone 51	1135	15

Approximately 3 × 10E7 cells in the logarithmic phase of growth were homogenized, supernatants places over a DEAE-cellulose, and eluates assayed as previously described (Kraft, 1983).

REFERENCES

Ashendel, C. The Phorbol Ester Receptor: a phospholipid-regulated protein kinase. (1984) Biochim. Biophys. Acta 822, 219–242.

Bollag, G. E., Roth, R. A., Beaudoin, J., Mochly—Rosen, D., Koshland, D. E. Jr. (1986) Protein kinase C directly phosphorylates the insulin receptor in vitro and reduces its protein-tyrosine kinase activity. Proc. Natl. Acad Sci. U.S.A. 83, pp 5822–4

Boreiko, C. , Mondal. S. , Narayan, S., and Heidel-berger, C. (1980) Effect of 12-O-Tetradecanoylphorbol-13-acetate on the Morphology and Growth of C3H/10T1/2 Mouse Embryo Cells. Cancer Res. 40, 4709–4716.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brandt, S. J., Niedel, J. E., Bell, R. M., Young, W. S. (1987) Distinct patterns of expression of different protein kinase C mRNAs in rat tissues. Cell 49, pp 57–63.

Catino, J. J., Francher, D. M., Edinger, K. J., and Steingfellow, D. A. (1985) A microtitre cytotoxicity assay useful for the discovery of fermentation-derived antitumor agents. Cancer Chemother. Pharmacol. 15, 240–243

Chomczynski. P., Sacchi, N. (1987), Anal. Biochem., 162: 156–159.

Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U., & Ullrich, A. (1986) Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signalling pathways. Science 233, 859–866.

Dailey, L., and Basilico, C. (1985). Sequences in the polyomavirus DNA regulatory region involved in viral DNA replication and early gene expression. J. Virol. 54, 739–749.

Daley, G. Q., McLaughlin, J., Witte, O. N., Baltimore, D. The CML-Specific P210 bcr/abl Protein, Unlike v-abl, Does Not Transform NIH/3T3 FibroblastsScience 237, pp 532–535.

Davis, R. J., and Czech, M. P. (1985) Platelet-derived growth factor mimics phorbol diester action on epidermal growth factor receptor phosphorylation at threonine 654. Proc. Natl. Acad. Sci. 82, 4080–4084.

Freeman, A. E., Price, P. J., Igel, H. J., Young, J. C., Maryak, J. M. Huebner, R. J. (1970) Morphological transformation of rat embryo cells induced by dimethylnitrosamine and murine leukemia viruses. J. Natl. Cancer Inst. 44, pp 65–78.

Freedman, V. H. and Shin, S. (1974) Cellular Tumorigenicity in nude Mice: Correlation with Cell Growth in Semi-Solid Medium. Cell 3, 355–359.

Graham, F. L., and van der Eb, A. J. (1973) A new technique for the assay of infectivity of human adenovirus DNA. Virology 52, 456–467.

Gould, K. L., Woodgett, J. R., Cooper, J. A., Buss, J. E., Shalloway, D., Hunter, T. (1985) Protein Kinase C Phosphorylates pp60src at a novel site. Cell 42, pp 849–857.

Horowitz, A. D., Greenebaum, E. and Weinstein, I. B. (1981) Identification of receptors for phorbol ester tumor promoters in intact mammalian cells and of an inhibitor of receptor binding in biologic fluids. Proc. Natl. Acad. Sci. U.S.A. 78, pp 2315–2319

Housey. G. M., O'Brian, C. A., Johnson, M. D., Kirschmeier. P., and Weinstein, I. B. (1987) Isolation of cDNA clones encoding protein kinase C: Evidence for a protein kinase C-related gene family. Proc. Natl. Acad. Sci. U.S.A. 84, pp 1065–1069

Housey, G. M., Kirschmeier, P., Garte, S. J., Burns, F., Troll, W., & Weinstein, I. B. (1985) Expression of long terminal repeat (LTR) sequences in carcinogen-induced murine skin carcinomas. Biochem. Biophys. Res. Commun. 127, 391–398.

Hsiao, W.-L. W., T. Wu, Wainstein, I. B., (1986) Oncogene-Induced Transformation of a Rat Embryo Fibroblast Cell Line is Enhanced by Tumor Promoters. Mol. Cell. Biol. 6, pp 1943–1950

Hunter, T., Ling, N., Cooper, J. A. (1984) Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature 311, 480–3

Huang, K. P., Nakabayashi, H., Huang, F. L. (1986) Isozymic forms of rat brain Ca2+-activated, phospholipid-dependent protein kinase. Proc. Natl. Acad. Sci. 83, 8535–8539.

Housey, G. M., Johnson, M. D., Hsiao, W. L., O'Brian, C. A., Murphy, J. P., Kirschmeier, P. and Weinstein, I. B., (1988), Cell, 52: 343–54.

Jaken, S. and Kiley, S. (1987) Purification and characterization of three types of protein kinase C from rabbit brain cytosol. Proc. Natl. Acad. Sci. U.S.A. 84, pp 4418–4422

Jeng, A. Y., Srivastava, S. K., Lacal, J. C., Blumberg, P. M. (1987) Phosphorylation of ras oncogene product by protein kinase C. Biochem. Biophys. Res. Commun. 145, pp 782–8.

Johnson, M. D., Housey, G. M., Kirschmeier, P., and Weinstein, I. B. (1987) Molecular Cloning of Gene Sequences Regulated by Tumor Promoters Through Protein Kinase C. Mol. Cell Biol. 7, 2821–2829

Kahn. C. R., White, M. F. (1988), The Insulin Receptor and the Molecular Mechanism of Insulin Action. J. Clin. Invest., 82: 1151–56.

Kajikawa, N., Kishimoto, A., Shiota, M., & Nishizuka, Y. (1983) Ca2+-dependent neutral protease and proteolytic activation of Ca2+-activated, phospholipid-dependent protein kinase. Methods. Enzymol. 102, 279–290.

Kawamoto, S. and Hidaka, H. (1984) 1-(5-Isoquinolinesulfonyl)-2-methyl-piperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. Biochem. Biophys. Res. Commun. 125, 258.

Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982) Calcium-activated, phospholipi-d-dependent protein kinase from rat brain. Subcellular distribution, purification, and properties. J. Biol. Chem. 257, 13341–13348.

Kirschmeier, P. T., Housey, G. M., Johnson, M. D., Perkins, A. S., and Weinstein, I. B. (1988) Construction and Characterization of a Retroviral Vector Demonstrating Efficient Expression of Cloned cDNA Sequences. DNA, in press.

Knopf, J. L., Lee, M.-H., Sultzman, L. A., Kriz, R. W., Loomis, C. R., Hewick, R. M. & Bell, R. (1986) Cloning and expression of multiple protein kinase C cDNAs. Cell 46, 491–502.

Kraft, A. S., Reeves, J. A. and Ashendel, C. L. (1988), J. Biol. Chem., 263: 8437–42.

Kraft and Anderson, W. B. (1983), J. Biol. Chem., 258: 9178–9183.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Leach, K. L., James, M. L., & Blumberg, P. M., (1983) Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol. Proc. Natl. Acad. Sci. U.S.A. 80, 4208–4212.

Lee, F., Yokota, T., Otsuka, T., Gemmell, L., Larson, N., Luh, J., Arai, K.-I., and Rennick, D. (1985), Proc. Nat. Acad. Sci. U.S.A., 82: 4360–64.

Lusky, M., and Botchan, M. (1981). Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences Nature 293 74–81.

Maddon, P. J., Dalgleish, A. G., McDougal, J. S., Clapham, P. R., Weiss, R. A., Axel, R. (1986) The T4 Gene Encodes the AIDS Virus Receptor and Is Expressed in the Immune System and the Brain. Cell 47, pp 333–348

Makowske, M., Birnbaum, M. J., Ballester, R., Rosen, O. M. (1986) A cDNA encoding PKC identifies two species of mRNA in brain and GH3 cells. J. Biol. Chem. 261, pp 13389–13392

Maniatis, T., Fritsch, E. F., & Sambrook, J. eds. (1983) in Molecular Cloning : A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.)

Mann, R., Mulligan, R. C., and Baltimore, D. (1983) Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus.' Cell 33,

Nishizuka, Y. (1986) Studies and Perspectives of Protein Kinase C. Science 233, 305–312.Nishizuka, Y. (1984) The Role of Protein Kinase C in Cell Surface Transduction and Tumour Promotion. Nature (London) 308, 693–698.

Metcalf, D., (1985) Science, 229, 16–22.

O'Brian, C. A., Lawrence, D. S., Kaiser, E. T., & Weinstein, I. B. (1984) Protein kinase C phosphorylates the synthetic peptide Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val in the presence of phospholipid plus either Ca2+ or a phorbol ester tumor promoter. Biochem. Biophys. Res. Commun. 124, 296–302.

O'Brian, C., Arcoleo, J., Housey, G. M., & Weinstein, I. B. (1985) in Cancer Cells 3, eds. Feramisco, J., Ozanne, B. & Stiles, C. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) 359–363.

O'Brian, C. A., Liskamp, R. M., Solomon, D. H. and Weinstein, I. B. (1985) Inhibition of Protein Kinase C by Tamoxifen. Cancer Res. 45, 2462–2465

Oho, Y., Kurokawa, T., Fujii, T., Kawahara, K., Igarashi, K., Kikkawa, U., Ogita, K., Nishizuka, Y. (1986) Two types of complementary DNAs of rat brain protein kinase C. FEBS 206. 347–52

Ono, Y., Kikkawa, U., Ogita, K., Tomoko, F., Kurokawa, T., Asaoka, Y, Sekiguchi, K., Ase, K., Igarashi. K., Nishizuka, Y. (1987) Expression and Properties of Two Types of Protein Kinase C: Alternative Splicing from a Single Gene. Science 236, pp 1116–1120.

Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokohura, H., Sakoh, T., Hidaka, H. (1987) Tissue-specific expression of three distinct types of rabbit protein kinase C. Nature (London) 325, pp 161–6.

Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., & Ullrich, A. (1986) The complete primary structure of protein kinase C—the major phorbol ester receptor. Science 233, 853–858.

Perkins, A. S., Kirschmeier, P. T., Gattoni-Celli, S., and Weinstein, I. B. (1983). Design of a retrovirus-derived vector for expression and transduction of exogenous genes in mammalian cells. Mol. Cell. Biol. 3, 1123–1132.

Pontremoli, S., Melloni, E., Michetti, M., Sparatore, B., Salamino, F., Sacco, O., and Horecker, B. L. (1987) Phosphorylation and proteolytic modification of specific cytoskeletal proteins in human neutrophils stimulated by phorbol-12-myristate 13-acetate. Proc. Natl. Acad. Sci. 84, 3604–3608.

Sibley, D. R., Benovic, J. L., Caron, M. G., Lefkowitz, R. J. (1987) Regulation of transmembrane signalling by receptor phosphorylation. Cell 48, 913–922.

Stabel, S., Rodriguez-Pena, A., Young S., Rozengurt E., and Parker, P. J. (1987), J. Cell Physiol., 130: 111–117.

Uehara, Y., Hori, M., Takeuchi, T., Umezawa, H. (1985) Screening of Agents Which Convert 'Transformed Morphology' of Rous Sarcoma Virus-Infected Rat Kidney Cells to 'Normal Morphology': Identification of an Active Agent as Herbimycin and its Inhibition of Intracellular src Kinase. Jpn. J. Cancer Res. 76, 672–675.

Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M.D., and Seeburg, P. H. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309, 418–425.

Walton, G. M., Bertics, P. J., Hudson, L. G., Vedvick, T. S., Gill, G. N. (1987) A Three-Step Purification Procedure for Protein Kinase C: Characterization of the Purified Enzyme. Anal. Biochem. 161, 425–437.

Weinstein, I. B., (1987) Growth Factors, Oncogenes, and Multistage Carcinogenesis. J. Cell. Biochem. 33, pp 213–224.

White, M. F., Livingston, J. N., Backer, J. M., Leuris, V., Dull, T. J., Ullrich, A., Kahn, C. R., Mutation of the Insulin Receptor at Tyrosine 960 Inhibits Signal Transmission but Does Not Affect its Tyrosine Kinase Activity (1988), Cell, 54: 641–649.

Wigler, M., Silverstien, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11, 223–232.

Woodgett, J. R., Gould, K. L., and Hunter, T. (1986) Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. Eur. J. Biochem. 161 177–184.

Yokota, T., Lee, F., Rennick, D., Hall, C., Aria, N., Mosmann, T., Nabel, G., Cantor, [illegible] and Arai, K.-I., (1984), Proc. Nat. Acad. Sci. USA 81: 1070–74.

Young, S., Parker, P. J., Ullrich, A., and Stabel, S. (1987) Down-regulation of protein kinase C is due to an increased rate of degradation. Biochem. J. 244, 775–779.

Von Hoff, D. D., Forseth, B., and Warfel, L. E. (1985) Use of a Radiometric System to Screen for Antineoplastic Agents: Correlation with a Human Tumor Cloning System. Cancer Res. 45, 4032–4036.

I claim:

1. A method of determining whether a substance is an inhibitor or an activator of a protein, which comprises:

a) providing a test cell which overproduces a selected protein relative to a control cell which produces said protein at a lower level or essentially does not produce the protein, and wherein production of said protein in said test cell evokes a responsive change in a phenotypic characteristic, other than the level of said protein in said cell, per se, which is comparatively greater than in said control cell,

b) treating said test cell containing the overproduced selected protein with said substance, and

c) examining the treated test cell to determine whether it exhibits a change in said phenotypic characteristic in response to said substance.

2. The process of claim 1 wherein the examination for a change in phenotypic characteristic in response to said substance includes comparing the response of the treated cell to the response of a comparable untreated test cell.

3. The method according to claim 1 wherein the examination includes comparing the phenotypic response of the treated test cell to that of a comparably treated test cell which does not overproduce the selected protein.

4. The method according to claim 1 wherein the test cell is selected from the group consisting of cells which have been genetically engineered by insertion of a nucleic acid sequence and cells which have been mutated to increase expression of the selected protein.

5. The method of claim 1 wherein the examination includes comparing the phenotypic response of the first test cell in the presence of the substance with the phenotypic response of the second test cell in the presence of a known inhibitor or activator of the protein.

6. The method of claim 1 wherein the change in a phenotypic characteristic is one observable with the naked eye.

7. The method of claim 1 wherein a change in a phenotypic characteristic is observed and said characteristic is selected from the group consisting of a cultural or morphological characteristic, the ability to grow in an anchorage independent fashion, the ability to grow on soft agar, foci formation in cell culture, the ability of the cell to take up a selected strain, the differentiation state of the cell, and an antigenic characteristic.

8. The method of claim 1 wherein the protein is selected from the group consisting of an enzyme, the expression product of an oncogene, an insulin receptor and a serotonin receptor.

9. The method of claim 1 wherein said test cell is obtained by introducing a gene encoding the protein of interest into a host cell, said gene being under the control of a promoter functional in the host cell, whereby said gene is expressed.

10. The method of claim 9 wherein the gene is introduced into the host cell by means of a genetic vector into which the gene has been inserted.

11. The method of claim 9 wherein the gene is introduced into the host cell by means of a retroviral vector.

12. The method of claim 9 in which the host cell essentially does not produce the protein.

13. The method of claim 9 in which the host cell is selected from the group consisting of a rat 6 fibroblast cell and a CHO cell.

14. The method according to claim 1 wherein the responsive change in a phenotypic characteristic is determined on a subcellular fraction of the test cell.

15. A method of determining whether a substance is an inhibitor or an activator of a protein, which comprises:

a) providing a first test cell which overproduces said protein relative to a control cell which produces said protein at a lower level or essentially does not produce the protein, and wherein production of said protein in said test cell exhibits a phenotypic characteristic other than the level of said protein in said cell, per se, in response to the protein which is comparatively greater than in said control cell;

b) incubating the substance with the first test cell;

c) comparing the phenotypic response of the first test cell in the presence of the substance with the phenotypic response of a second test cell in the absence of the substance.

16. The method of claim 15 wherein said first and second cell are derived from the same line cell.

* * * * *

US005877007A

United States Patent [19]

Housey

[11] Patent Number: 5,877,007

[45] Date of Patent: Mar. 2, 1999

[54] METHOD OF SCREENING FOR PROTEIN INHIBITORS AND ACTIVATORS

[75] Inventor: Gerard Housey, New York, N.Y.

[73] Assignee: ICT Pharmaceuticals, Inc., New York, N.Y.

[21] Appl. No.: 473,169

[22] Filed: Jun. 7, 1995

Related U.S. Application Data

[62] Division of Ser. No. 408,443, Mar. 17, 1995, which is a continuation of Ser. No. 977,986, Nov. 18, 1992, abandoned, which is a continuation of Ser. No. 392,073, Aug. 10, 1989, Pat. No. 5,266,464, which is a continuation-in-part of Ser. No. 154,206, Feb. 10, 1988, Pat. No. 4,980,281.

[51] Int. Cl.[6] C12N 1/21; C12N 1/00; C12P 21/02; G01N 33/573

[52] U.S. Cl. 435/252.3; 435/7.4; 435/7.71; 435/29; 435/69.1

[58] Field of Search 435/6, 7.4, 7.71, 435/69.1, 183, 252.3, 29, 240.1, 240.2

[56] References Cited

U.S. PATENT DOCUMENTS

4,980,281 12/1990 Housey 435/29

4,981,790 1/1991 Haseltine et al. 435/69.1

5,266,464 11/1993 Housey 435/29

Primary Examiner—Nancy Degen

Attorney, Agent, or Firm—Kenyon & Kenyon

[57] ABSTRACT

Cells useful in the pharmacological characterization of inhibitors or activators of proteins are provided. These cells a protein, the presence of which affects a particular characteristic of the cell which changes in response to substances which inhibit or activate the protein. Such responsive changes in a phenotypic characteristic may be utilized in many useful ways, including the discovery, development or characterization of substances suitable for the treatment of diseases or other conditions in human beings or animals. Such cells may also be useful for studying diseases or other biological processes, for determining the effects of various drugs alone or in combination, as well as for identifying or characterizing substances which may be useful in reducing or preventing the occurrence of a disease or other condition. Through the use of such cells in a cell based assay, tamoxifen is identified as an inhibitor of Protein Kinase C (PKC) activity in cell culture.

1 Claim, 22 Drawing Sheets

FIG. 1.A.- 1

```
          10          20          30          40          50          60
          *           *           *           *           *           *
GAA TTC CGC CTC TCC GGG CTT ACA GCC CGC GGT CCC GCC GCC CCG GGG CCG CCA CCT CTC

          70          80          90          100         110         120
          *           *           *           *           *           *
GGG GCT CCC CCC AGT CCC CGC GCG CGC AAG ATG GCT GAC CCG GCT GCG GGG CCG CCG CCG
                                    Met Ala Asp Pro Ala Ala Gly Pro Pro Pro

          130         140         150         160         170         180
          *           *           *           *           *           *
AGC GAG GGC GAG GAG AGC ACG GTG CGC TTC GCC CGC AAA GGG CCC CTC CGG CAG AAG AAC
Ser Glu Gly Glu Glu Ser Thr Val Arg Phe Ala Arg Lys Gly Pro Leu Arg Gln Lys Asn
```

FIG. 1.A. - 2

```
        190         200         210         220         230         240
         *           *           *           *           *           *
GTG CAC GAG GTG AAG AAC CAC AAA TTC ACC GCC CGC TTC TTC AAG CAG CCC ACC TTC TGC
Val His Glu Val Lys Asn His Lys Phe Thr Ala Arg Phe Phe Lys Gln Pro Thr Phe Cys

        250         260         270         280         290         300
         *           *           *           *           *           *
AGC CAC TGC ACC GAC TTC ATT TGG GGC TTC GGG AAG CAG GGA TTC CAG TGT CAA GTC TGC
Ser His Cys Thr Asp Phe Ile Trp Gly Phe Gly Lys Gln Gly Phe Gln Cys Gln Val Cys

        310         320         330         340         350         360
         *           *           *           *           *           *
TGC TTT GTT GTA CAC AAG CGC TGC CAT GAA TTC GTC ACG TTC TCC TGC CCT GGT GCA GAC
Cys Phe Val Val His Lys Arg Cys His Glu Phe Val Thr Phe Ser Cys Pro Gly Ala Asp
```

FIG. 1.A. - 3

```
          370         380         390         400         410         420
           *           *           *           *           *           *
AAG GGC CCG GCC TCT GAT GAC CCA CGG AGC AAA CAC AAG TTT AAG ATC CAC ACC TAC TCC
Lys Gly Pro Ala Ser Asp Asp Pro Arg Ser Lys His Lys Phe Lys Ile His Thr Tyr Ser

          430         440         450         460         470         480
           *           *           *           *           *           *
AGC CCT ACC TTC TGT GAC CAC TGT GGA TCA CTG CTG TAT GGG CTC ATC CAC CAG GGG ATG
Ser Pro Thr Phe Cys Asp His Cys Gly Ser Leu Leu Tyr Gly Leu Ile His Gln Gly Met

          490         500         510         520         530         540
           *           *           *           *           *           *
AAA TGC GAC ACC TGT ATG ATG AAT GTC CAC AAG CGC TGC GTG ATG AAC GTC CCC AGC CTC
Lys Cys Asp Thr Cys Met Met Asn Val His Lys Arg Cys Val Met Asn Val Pro Ser Leu
```

FIG. 1.A. - 4

```
          550         560         570         580         590         600
           *           *           *           *           *           *
TGT GGC ACC GAC CAC ACA GAA CGC CGT GGC CGC ATC TAC ATC CAG GCC CAC ATC GAC AGG
Cys Gly Thr Asp His Thr Glu Arg Arg Gly Arg Ile Tyr Ile Gln Ala His Ile Asp Arg

          610         620         630         640         650         660
           *           *           *           *           *           *
GAG GTC CTC ATC GTT GTT GTA AGA GAT GCT AAA AAT CTG GTA CCT ATG GAC CCC AAC GGC
Glu Val Leu Ile Val Val Val Arg Asp Ala Lys Asn Leu Val Pro Met Asp Pro Asn Gly

          670         680         690         700         710         720
           *           *           *           *           *           *
TTG TCA GAT CCC TAC GTA AAA CTG AAA CTG ATC CCT GAT CCC AAA AGT GAG AGC AAG CAG
Leu Ser Asp Pro Tyr Val Lys Leu Lys Leu Ile Pro Asp Pro Lys Ser Glu Ser Lys Gln
```

FIG. 1.A. - 5

```
      730         740         750         760         770         780
       *           *           *           *           *           *
AAG ACC AAG ACT ATC AAA TGC TCC CTC AAC CCG GAG TGG AAC GAA ACC TTC AGA TTT CAG
Lys Thr Lys Thr Ile Lys Cys Ser Leu Asn Pro Glu Trp Asn Glu Thr Phe Arg Phe Gln

      790         800         810         820         830         840
       *           *           *           *           *           *
CTG AAG GAA TCA GAC AAA GAC AGA AGA CTG TCC GTA GAG ATC TGG GAT TGG GAC CTG ACC
Leu Lys Glu Ser Asp Lys Asp Arg Arg Leu Ser Val Glu Ile Trp Asp Trp Asp Leu Thr

      850         860         870         880         890         900
       *           *           *           *           *           *
AGC AGG AAT GAC TTC ATG GGA TCT CTG TCG TTT GGG ATT TCA GAA CTA CAG AAA GCC GGA
Ser Arg Asn Asp Phe Met Gly Ser Leu Ser Phe Gly Ile Ser Glu Leu Gln Lys Ala Gly
```

FIG. 1.A. - 6

```
        910         920         930         940         950         960
         *           *           *           *           *           *
GTG GAT GGC TGG TTC AAG TTA CTA AGC CAG GAA GAA GGC GAG TAC TTT AAT GTG CCG GTG
Val Asp Gly Trp Phe Lys Leu Leu Ser Gln Glu Glu Gly Glu Tyr Phe Asn Val Pro Val

        970         980         990        1000        1010        1020
         *           *           *           *           *           *
CCG CCG GAA GAA AGC GAG GGC AAT GAA GAG CTG CGG CAG AAG TTT GAG AGA GCC AAG ATT
Pro Pro Glu Glu Ser Glu Gly Asn Glu Glu Leu Arg Gln Lys Phe Glu Arg Ala Lys Ile

       1030        1040        1050        1060        1070        1080
         *           *           *           *           *           *
GGC CAA GGT ACC AAG GCT CCA GAA GAA AAG ACA GCG AAC ACT ATA TCC AAA TTT GAC AAC
Gly Gln Gly Thr Lys Ala Pro Glu Glu Lys Thr Ala Asn Thr Ile Ser Lys Phe Asp Asn
```

FIG. 1.A. - 7

```
      1090        1100        1110        1120        1130        1140
       *           *           *           *           *           *
AAT GGC AAC AGG GAC CGG ATG AAA CTG ACC GAT TTT AAC TTC CTG ATG GTG CTG GGG AAA
Asn Gly Asn Arg Asp Arg Met Lys Leu Thr Asp Phe Asn Phe Leu Met Val Leu Gly Lys

      1150        1160        1170        1180        1190        1200
       *           *           *           *           *           *
GGC AGC TTT GGC AAG GTC ATG CTC TCA GAG CGG AAG GGT ACA GAT GAA CTC TAT GCC GTG
Gly Ser Phe Gly Lys Val Met Leu Ser Glu Arg Lys Gly Thr Asp Glu Leu Tyr Ala Val

      1210        1220        1230        1240        1250        1260
       *           *           *           *           *           *
AAG ATC CTG AAG AAA GAT GTG GTG ATC CAA GAT GAC GAT GTG GAG TGC ACA ATG GTG GAG
Lys Ile Leu Lys Lys Asp Val Val Ile Gln Asp Asp Asp Val Glu Cys Thr Met Val Glu
```

FIG. 1.A. - 8

```
      1270        1280        1290        1300        1310        1320
       *           *           *           *           *           *
AAG AGG GTG CTG GCC CTG CCT GGG AAG CCC CCA TTC CTG ACT CAG CTC CAT TCC TGC TTC
Lys Arg Val Leu Ala Leu Pro Gly Lys Pro Pro Phe Leu Thr Gln Leu His Ser Cys Phe

      1330        1340        1350        1360        1370        1380
       *           *           *           *           *           *
CAG ACC ATG GAC CGC CTC TAC TTT GTG ATG GAG TAT GTG AAC GGG GGC GAC CTC ATG TAC
Gln Thr Met Asp Arg Leu Tyr Phe Val Met Glu Tyr Val Asn Gly Gly Asp Leu Met Tyr

      1390        1400        1410        1420        1430        1440
       *           *           *           *           *           *
CAC ATC CAA CAA GTT GGC CGT TTC AAG GAG CCC CAT GCT GTA TTT TAC GCT GCA GAG ATT
His Ile Gln Gln Val Gly Arg Phe Lys Glu Pro His Ala Val Phe Tyr Ala Ala Glu Ile
```

121b

FIG. 1.A. - 9

```
      1450        1460        1470        1480        1490        1500
       *           *           *           *           *           *
GCC ATC GGT CTT TTC TTC TTG CAG AGC AAG GGC ATC ATT TAC CGT GAC CTG AAA CTT GAC
Ala Ile Gly Leu Phe Phe Leu Gln Ser Lys Gly Ile Ile Tyr Arg Asp Leu Lys Leu Asp

      1510        1520        1530        1540        1550        1560
       *           *           *           *           *           *
AAC GTG ATG CTG GAT TCC GAG GGG CAC ATC AAA ATC GCT GAC TTT GGC ATG TGT AAA GAG
Asn Val Met Leu Asp Ser Glu Gly His Ile Lys Ile Ala Asp Phe Gly Met Cys Lys Glu

      1570        1580        1590        1600        1610        1620
       *           *           *           *           *           *
AAT ATC TGG GAT GGG GTG ACA ACC AAG ACA TTC TGT GGC ACT CCA GAC TAC ATT GCC CCA
Asn Ile Trp Asp Gly Val Thr Thr Lys Thr Phe Cys Gly Thr Pro Asp Tyr Ile Ala Pro
```

FIG. 1.A. - 10

```
      1630        1640        1650        1660        1670        1680
       *           *           *           *           *           *
GAG ATC ATT GCT TAT CAG CCC TAC GGA AAG TCT GTG GAC TGG TGG GCG TTT GGA GTC CTG
Glu Ile Ile Ala Tyr Gln Pro Tyr Gly Lys Ser Val Asp Trp Trp Ala Phe Gly Val Leu

      1690        1700        1710        1720        1730        1740
       *           *           *           *           *           *
CTG TAT GAA ATG TTG GCT GGC CAG GCA CCT TTT GAA GGG GAG GAT GAG GAT GAA CTC TTC
Leu Tyr Glu Met Leu Ala Gly Gln Ala Pro Phe Glu Gly Glu Asp Glu Asp Glu Leu Phe

      1750        1760        1770        1780        1790        1800
       *           *           *           *           *           *
CAG TCA ATC ATG GAG CAC AAC GTG GCG TAT CCC AAG TCC ATG TCT AAG GAA GCT GTG GCA
Gln Ser Ile Met Glu His Asn Val Ala Tyr Pro Lys Ser Met Ser Lys Glu Ala Val Ala
```

123b

FIG. 1.A. - 11

```
      1810        1820        1830        1840        1850        1860
       *           *           *           *           *           *
ATC TGC AAA GGG CTA ATG ACC AAA CAC CCA GGC AAG CGC CTG GGT TGT GGG CCT GAA GGG
Ile Cys Lys Gly Leu Met Thr Lys His Pro Gly Lys Arg Leu Gly Cys Gly Pro Glu Gly

      1870        1880        1890        1900        1910        1920
       *           *           *           *           *           *
GAA CGA GAC ATT AAG GAG CAT GCA TTT TTC CGG TAT ATC GAC TGG GAG AAA CTC GAA CGC
Glu Arg Asp Ile Lys Glu His Ala Phe Phe Arg Tyr Ile Asp Trp Glu Lys Leu Glu Arg

      1930        1940        1950        1960        1970        1980
       *           *           *           *           *           *
AAG GAG ATT CAG CCA CCT TAT AAA CCA AAA GCT AGA GAC AAG CGA GAC ACC TCC AAC TTC
Lys Glu Ile Gln Pro Pro Tyr Lys Pro Lys Ala Arg Asp Lys Arg Asp Thr Ser Asn Phe
```

FIG. 1.A. - 12

```
        1990        2000        2010        2020        2030        2040
         *           *           *           *           *           *
GAC AAA GAG TTC ACC AGG CAG CCT GTG GAA CTG ACT CCC ACT GAC AAA CTC TTC ATC ATG
Asp Lys Glu Phe Thr Arg Gln Pro Val Glu Leu Thr Pro Thr Asp Lys Leu Phe Ile Met

        2050        2060        2070        2080        2090        2100
         *           *           *           *           *           *
AAC TTG GAC CAA AAT GAA TTT GCT GGC TTC TCG TAT ACT AAC CCA GAG TTT GTC ATT AAT
Asn Leu Asp Gln Asn Glu Phe Ala Gly Phe Ser Tyr Thr Asn Pro Glu Phe Val Ile Asn

        2110        2120        2130        2140        2150        2160
         *           *           *           *           *           *
GTG TAG GTG AAT GCA GAT TCC ATC GCT GAG CCT GTG TGT AAG GCT GCA GCG TGA ATG TCT
Val ---
```

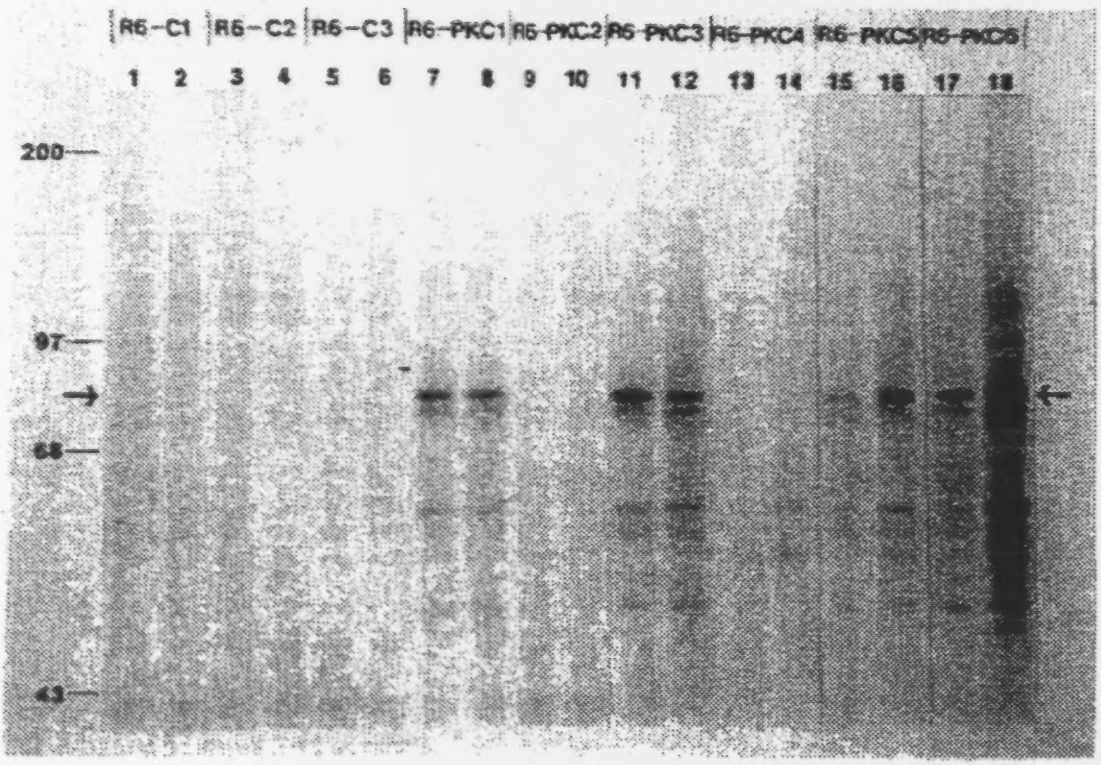
FIG. A.1. - 13

```
      2170        2180        2190        2200        2210        2220
       *           *           *           *           *           *
ATT ATC AAT TCC AGT CTT CCA GGA TTC ATG GTG CCT CTG TTG GCA TCC GTC ATG TGG AGA

      2230        2240        2250        2260        2270        2280
       *           *           *           *           *           *
GCT TGT CTT AGA GGG CTT TTC TTT GTA TGT ATA GCT TGC TAG TTT GTT TTC TAC ATT TCA

      2290        2300        2310        2320        2330        2340
       *           *           *           *           *           *
AAA TGT TTA GTT TAG AAT AAG TGC ATT GCC CAC TGA TAG AGG TAC AAT TTT CCA GAC TTC
```

FIG. 1.A. - 15

```
      2530        2540        2550        2560        2570        2580
       *           *           *           *           *           *
GTA AGC TTT GCA GTT ACT GTG AAC TCT TGT CTC TTG GAG GAA CTT TTT GTT TAA GAA TTG

      2590        2600
       *           *
GTA TGA TTA AAC TGA ATT C*
```

FIG. 2



BEST AVAILABLE COPY

FIG. 3

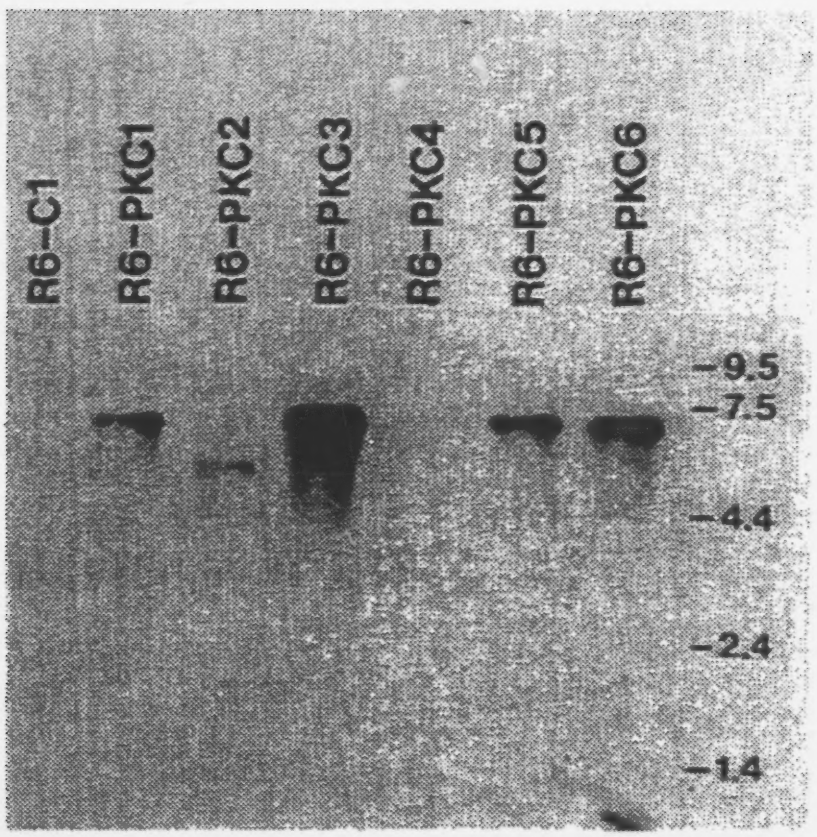

R6–C1
R6–PKC1
R6–PKC2
R6–PKC3
R6–PKC4
R6–PKC5
R6–PKC6
–9.5
–7.5
–4.4
–2.4
–1.4

FIG. 4

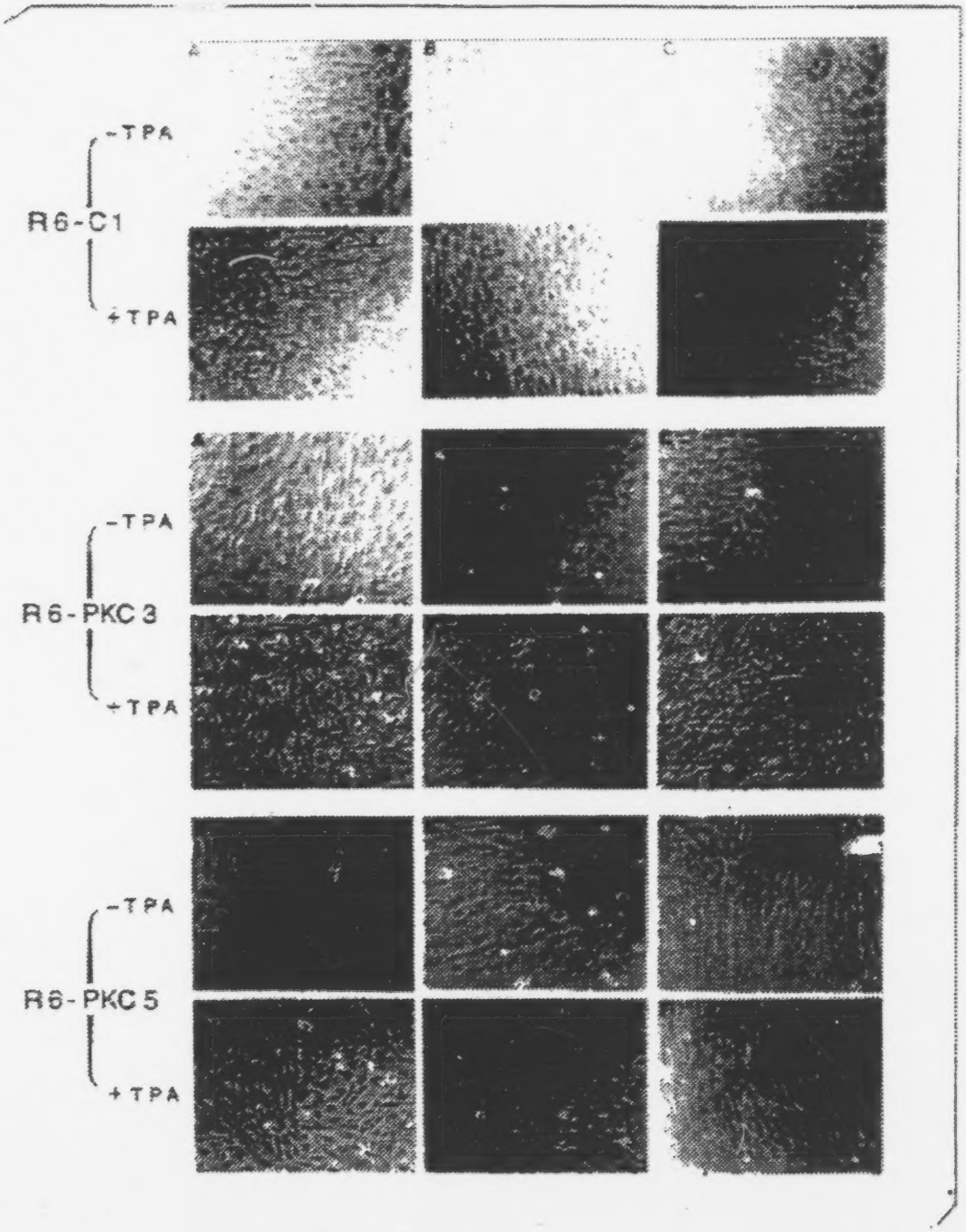


FIG. 5.

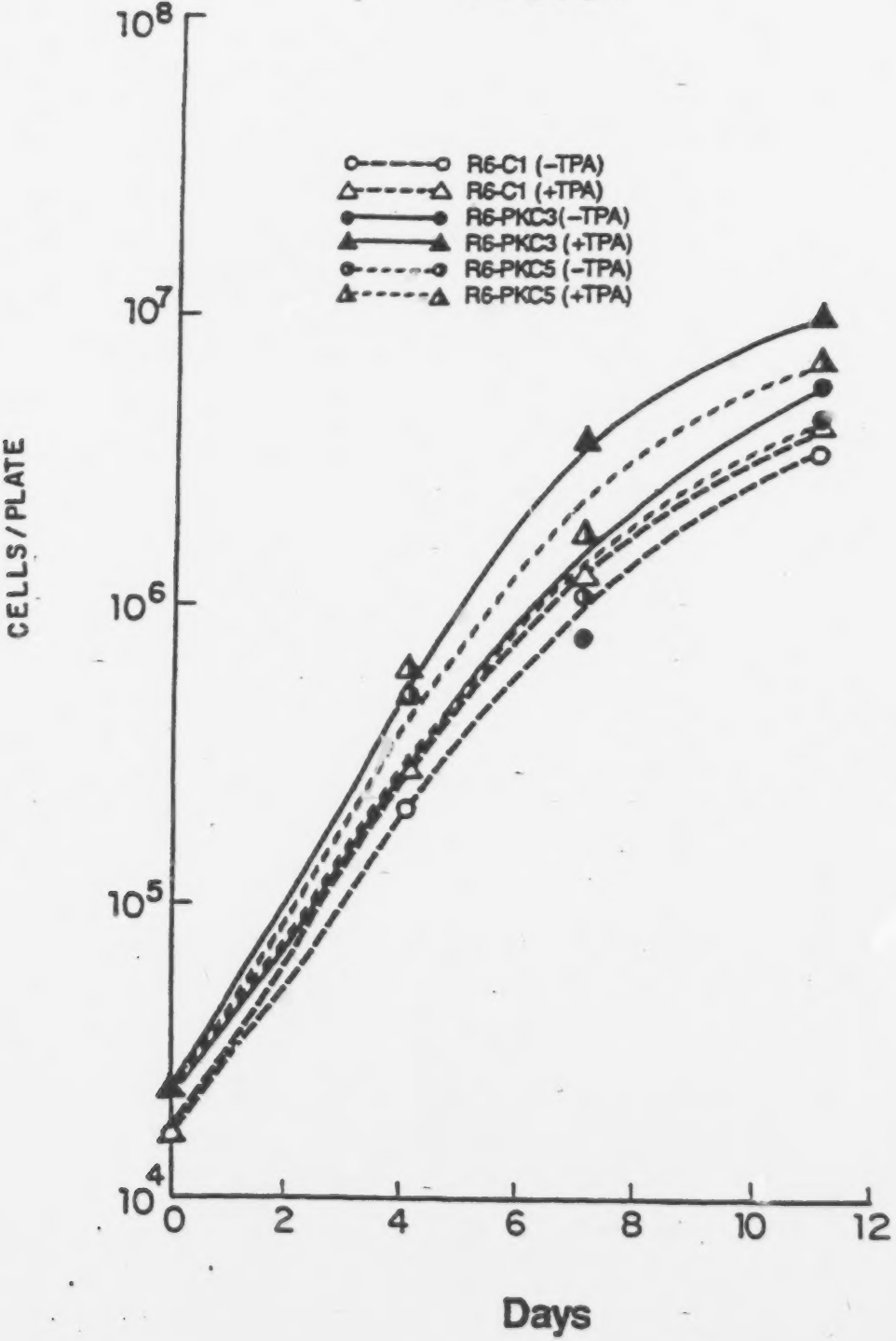

R6-C1 (–TPA)
R6-C1 (+TPA)
R6-PKC3(–TPA)
R6-PKC3 (+TPA)
R6-PKC5 (–TPA)
R6-PKC5 (+TPA)
CELLS/PLATE
10^8
10^7
10^6
10^5
10^4
0
2
4
6
8
10
12
Days

FIG. 6

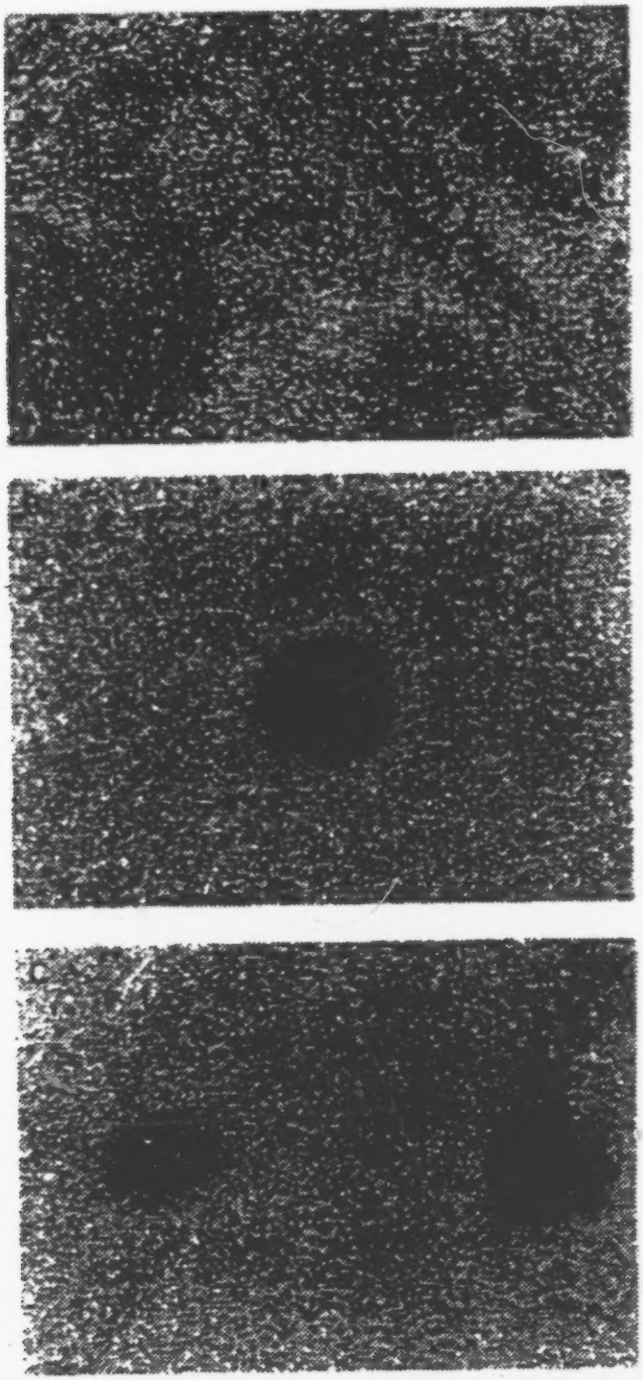
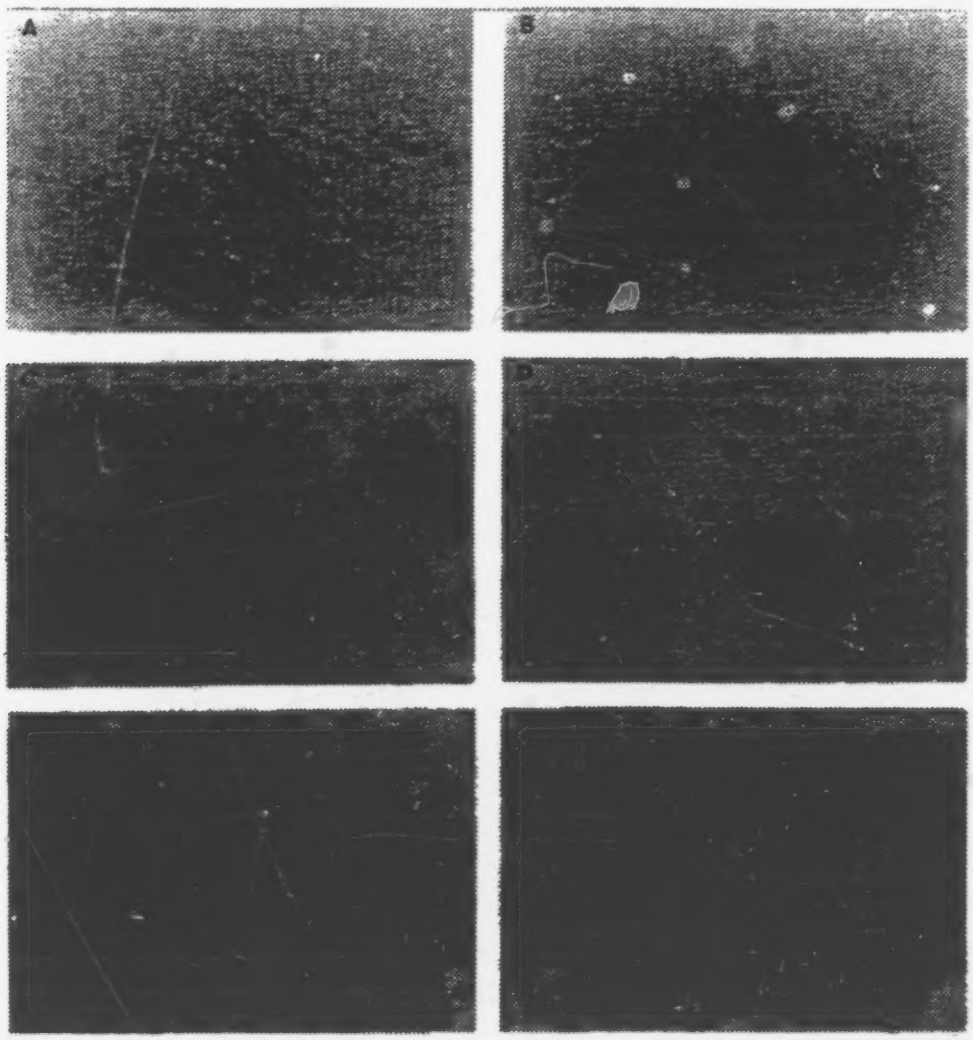


FIG. 7



1

METHOD OF SCREENING FOR PROTEIN INHIBITORS AND ACTIVATORS

This application is a divisional application of U.S. Ser. No. 08/408,443, filed 17 Mar. 1995, which is a continuation of U.S. Ser. No. 07/977,986, filed 18 Nov. 1992, now abandoned, which is a continuation of U.S. Ser. No. 07/392,073, filed 10 Aug. 1989, now U.S. Pat. No. 5,266,464, which is a continuation-in-part of U.S. Ser. No. 07/154,206, filed 10 Feb. 1988, now U.S. Pat. No. 4,980,281 the benefit of whose filing date is claimed pursuant to 35 U.S.C. § 120.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a general screening method for the discovery and identification of both inhibitors and activators of enzymes, receptors, and other proteins. In particular, it is concerned with a method of screening for substances which specifically inhibit or activate a particular protein affecting the cultural or morphological characteristics of the cell expressing the protein, especially in a manner apparent to the naked eye.

2. Information Disclosure Statement

A number of assay systems are currently in use for the discovery of new modulators of cell growth, and in particular, in the search for new anti-cancer drugs which are specifically toxic to cancer cells but not to normal cells. A variety of changes may be scored for, but the most common ones are reversion of the transformed phenotype, significant changes in cell morphology, or cytotoxicity. The assays include: (1) in vitro cytotoxicity assays; (2) soft agar colony formation assays; (3) in vitro anti-microbial assays; and (4) assays which detect changes in cellular morphology.

In vitro cytotoxicity assays involve the measurement of cellular parameters which are indicative of inhibition of cellular growth or cytotoxicity. These include, for example, the measurement of the inhibition of certain cellular metabolic pathways in response to treatment with cytotoxic agents. The papers by Von Hoff, et al. (1985), and Catino, et al. (1985) describe typical methods which use this technique. These methods are somewhat complex technically, and require the use of radioactive tracers in some cases. Furthermore, the results are non-specific since any agent which alters the growth properties of cells will score positively in these assay systems.

Agents have also been tested for their ability to inhibit transformed (cancerous) cells from growing in soft agar. This method is based upon the finding by Freedman and Shin (1974) that the formation of colonies of cells in soft agar is the in vitro test which shows the highest correlation in predicting whether the cells will be tumorigenic in an experimental animal. This method is relatively simple to perform since colony growth will, after two or more weeks, generally be large enough to be seen with the naked eye. Scoring the final results, therefore, can be performed either by a technician without extensive training in tissue culture, or, as we describe in the current application, by an automated absorbance detection system. In its present form, however, this method is also non-specific for the same reasons as described above. In other words, any agent which inhibits cellular growth in any way will scores positively in this assay system as it is currently used, whether or not it inhibits the protein of interest.

In vitro anti-microbial assays involve the use of bacterial or yeast strains which are used as test organisms for screening for agents with generalized growth inhibitory properties

2

(also described in Catino, et al., 1985) In this method, the bacterial or yeast strain is grown on standard media plates and potential agents are applied to various spots on the plates. If an agent has growth inhibitory properties, a clear zone results at the site of its application on the plate, resulting from the inability of the test strain to grow in the area. This method is rapid and can be performed by a technician without extensive training in tissue culture techniques, but the results are generally non-specific because agents which are effective against bacterial or yeast strains are frequently less effective (or completely ineffective) in modulating the growth of mammalian cells, as shown in the paper by Catino et al. (1985).

Still other screening systems depend upon a morphologic alteration of the test cells by exposure to the potential agents in order to determine the effectiveness of a given agent. This method is currently the most effective one for developing specific agents which interact with a given protein or alter a specific cellular property, as evidenced by the representative paper by Uehara, et. al. (1985). However, these screening systems are the most difficult ones to apply in practice, since the morphologic effect of each individual agent on the test cells must be studied under the microscope. Hence this method requires extensive observations of the cells by a trained scientist.

SUMMARY OF THE INVENTION

The Method presented in detail in this application combines the rapidity and ease of performance of the soft agar assay with a specificity for detecting an active agent exceeding that of the morphology assay. In brief the method which we describe herein involves the generation of a cell line purposefully engineered to detect both stimulatory and inhibitory agents which are absolutely specific for any given protein which affects the cultural or morphological characteristics of the cell.

The basis for this invention is my observation that if a protein (the "protein of interest", or POI) which is involved in some manner in cellular growth control is overproduced in cells, then pharmacologic agents which can activate or inhibit the POI can result in altered growth properties of the cells.

The sensitivity of the cells is dependent on their production of the POI, a phenomenon referred to herein as a "graded cellular response" to the pharmacologically active agent.

The present invention provides a rapid, yet powerful screening system for the discovery and identification of both inhibitors and activators of proteins. The method may be applied to virtually any type of protein, including enzymes, receptors, DNA- or RNA-binding proteins, or others which are directly or indirectly involved in regulating cellular growth.

The method involves the insertion of a DNA (or cDNA) sequence encoding the Protein Of Interest (POI) into an appropriate vector and the generation of cell lines which contain either (1) the expression vector alone ("control" cell lines) or (2) the expression vector containing the inserted DNA (or cDNA) sequence encoding the POI ("test" cell lines). Using the appropriate vector system, recipient cell lines, and growth conditions, test cell lines can thus be generated which stably overproduce the corresponding POI. Under the appropriate growth conditions, these cell lines will exhibit a "graded cellular response" to activators or inhibitors of the POI. A screening system can thus be set up whereby the control and test cell lines are propagated in

defined growth conditions in tissue culture dishes (or even in experimental animals) and large numbers of compounds (or crude substances which may contain active compounds) can be screened for their effects on the POI.

Substances which inhibit or activate the POI may affect characteristics such as growth rate, tumorigenic potential, anti-tumorigenic potential, anti-metastatic potential, cell morphology, antigen expression, and/or anchorage-independent growth capability. Substances which specifically inhibit or inactivate the POI may be distinguished from substances which affect cell morphology or growth by other mechanisms in that they will have a greater effect on the test lines than on the control lines.

The system has been tested using several cDNA sequences and several recipient cell lines, and can be easily automated.

The appended claims are hereby incorporated by reference as an enumeration of the preferred embodiments. All references cited anywhere in this specification are hereby incorporated by reference to the extent pertinent.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows the full-length cDNA sequence, and the deduced amino acid sequence, of one of several forms of PKC which has previously been isolated (cDNA clone RP58), and whose partial sequence has been reported (Housey, et al., 1987). It corresponds to PKCbeta1 according to the nomenclature of Ono, et al. (1987). The deduced amino acid sequence begins with the first in-frame methionine codon at nucleotide position 91 and encodes a 671 amino acid protein with a predicted molecular weight of 76.8 kd. A consensus polyadenylation signal is underlined.

FIG. 1B shows the retrovirus-derived cDNA expression vector, developed in this laboratory, which was used for the present studies. The full-length RP58 cDNA encoding PKC-beta1 (shown in 1A) was cloned into the Eco RI site of plasmid pMV7. The shaded region represents the coding sequence. "E" and "P" designate Eco RI and Pst I restriction sites, respectively. The indicated sizes between restriction sites in the RP58 cDNA are given in kilobases. "LTR" designates the 5' (left) and 3' (right) long terminal repeats of Moloney murine leukemia virus, and "TK-neo" designates the promoter region of the HSV thymidine kinase gene linked to the 5' end of the bacterial neomycin phosphotransferase (neo) gene.

FIG. 1-C outlines in schematic form the overall strategy used to generate cell lines stably overproducing PKC.

FIG. 2. Purification and Autophosphorylation of PKC. PKC activity from each cell line was purified and subjected to reaction conditions favoring autophosphorylation of PKC. Following the autophosphorylation reaction, protein samples were separated by discontinuous polyacrylamide gel electrophoresis. In the lanes bearing odd numbers the reaction mixtures contained 1 mM Ca2+ and phosphatidylserine to activate PKC, and in the lanes bearing even numbers the reaction mixtures contained 1 mM EGTA, 100 ng/ml TPA, and phosphatidylserine. The numbers in the left margin indicate the sizes of molecular weight markers, in kd. Arrows indicate the position of the 75 kd autophosphorylated PKC.

FIG. 3. Northern Blot Hybridization Analyses. Poly A+ RNA was isolated from the indicated cell lines and separated by electrophoresis on 6% formaldehyde/1% agarose gels, blotted onto nylon membrane and hybridized with the 32P-labelled full-length RP58 cDNA probe, as previously described (Housey, et. al., 1987). The numbers in the right margin indicate the sizes in kb of the RNA markers. The R6-PKC4 sample displayed a very weak 4.8 kb band on the original autoradiograph.

FIG. 4. Morphologic Responses of the Cell Lines to Phorbol Ester Treatment. Nearly confluent cultures of the three indicated cell lines were exposed to 100 ng/ml TPA in 0.1 % dimethylsulfoxide (DMSO) solvent ("+TPA") or 0.1% DMSO alone ("−TPA"), in DMEM plus 10% CS. Photographs were taken 24 hours later (Panels A and D) and 48 hours later (Panels B and E). Fresh medium plus or minus TPA was then added and photographs were then taken an additional 24 hours later (Panels C and F). (Magnification: 100×).

FIG. 5. Growth Curves of Control and PKC-Overproducing Cell Lines. The indicated cell lines were seeded at 1×10^4 per 6 cm plate in DMEM plus 10% CS, in the presence ("+TPA") and absence ("−TPA") of 100 ng/ml TPA. Cell numbers were determined in replicate plates during the subsequent 11 day growth period. The values given indicate the means of triplicate determinations, which varied by less than 10%.

FIG. 6. Post-confluence Foci Formation. Control R6-C2 cells (Panel A) and R6-PKC3 cells (Panels B and C) were grown to confluence and then maintained for an additional 28 days in DMEM plus 10% CS (without TPA), with the addition of fresh medium every 3 days. Photographs were taken at the end of the 28 day period. Magnification: 40×.

FIG. 7. Growth in Soft Agar. Cell lines R6-C1, R6-PKC3, and R6-PKC5 were seeded into 60 mm petri dishes in 0.3% agar containing DMEM plus 20% FBS and 50 lg/ml G418, plus or minus 100 ng/ml TPA. Photographs were taken after 21 days of growth. A) R6-C1+TPA (low-power field) B) R6-C1+TPA (medium-power field) C) R6-PKC3 D) R6-PKC3+TPA E) R6-PKC5 F) R6-PKC5+TPA. For additional details see Experimental Procedures. (Magnification: 100× in panel B; 40× in all other panels).

DETAILED DESCRIPTION OF THE INVENTION

The present method is intended for use in identifying potential chemical inhibitors or activators of enzymes, receptors, or any proteins which have effects upon cell phenotype. This method requires two cell lines, preferably alike except for their expression (production) of the protein of interest at different levels (and any further differences necessitated by that difference in expression). Inhibitors or activators are identified by their greater effect on the phenotype of the higher producing cell line.

Any phenotypic characteristic of the cell which is affected by expression of the protein of interest, other, of course, than the level of the protein itself, may be assayed. The phenotypic characteristic is preferably a "cultural" or "morphological" characteristic of the cell. For purposes of the appended claims, these terms are defined as follows:

Cultural characteristics include, but are not limited to the nutrients required for growth, the nutrients which, though not required for growth, markedly promote growth, the physical conditions (temperature, pH, gaseous environment, osmotic state, and anchorage dependence or independence) of the culture which affect growth, and the substances which inhibit growth or even kill the cells.

Morphological characteristics, but are not limited to include the size and shape of cells, their arrangement, cell differentiation, and subcellular structures.

Where the protein of interest is implicated in tumorigenesis or related phenomena, the characteristic observed is

FIG. 1.A. - 14

```
        2350        2360        2370        2380        2390        2400
         *           *           *           *           *           *
CAG AAA CTC ATC CAA TGA ACC AAC AGT GTC AAA ACT TAA CTG TGT CCG ATA CCA AAA TGC

        2410        2420        2430        2440        2450        2460
         *           *           *           *           *           *
TTC AGT ATT TGT AAT TTT TAA AGT CAG ATG CTG ATG TTC CTG GTC AAA GTT TTT ACA GTT

        2470        2480        2490        2500        2510        2520
         *           *           *           *           *           *
ACT CTC GAA TAT CTC CTT TGA ATG CTA CCT AAG CAT GAC CGG TAT TTT TAA AAG TTG TGA
```

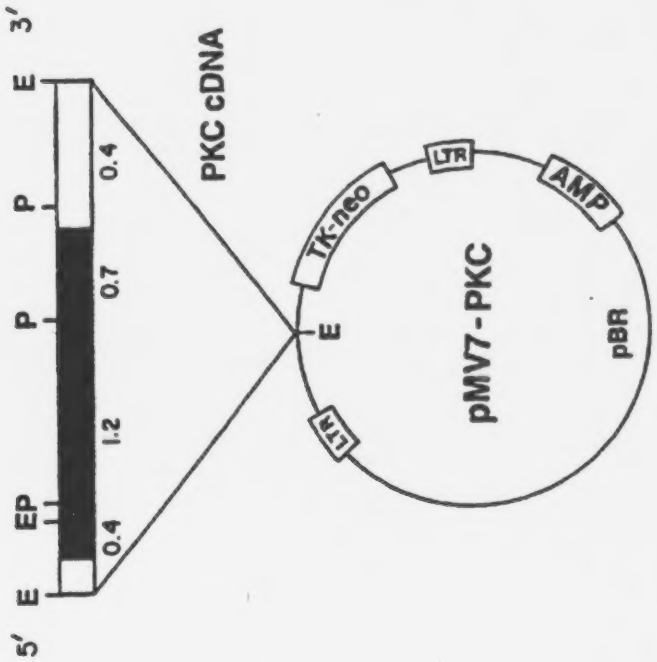

5'
E
EP
P
P
E
3'
1.2
0.7
0.4
0.4
PKC cDNA
pMV7-PKC
TK-neo
LTR
AMP
pBR
E
LTR

FIG. 1.
B.

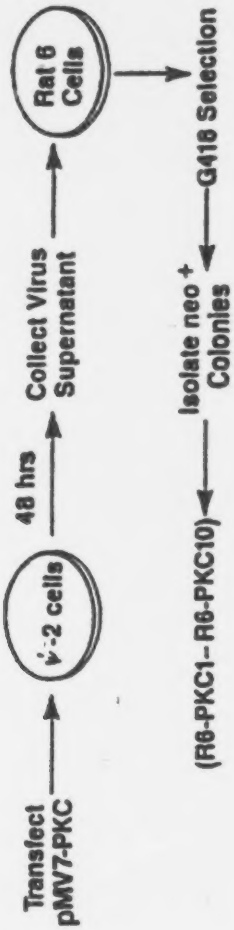

Transfect pMV7-PKC
Ψ-2 cells
48 hrs
Collect Virus Supernatant
Rat 6 Cells
G418 Selection
Isolate neo+ Colonies
(R6-PKC1 - R6-PKC10)

FIG. 1.
C.

preferably one related to cellular growth control, differentiation, de-differentiation, carcinogenic transformation, metastasis, tumorigenesis, or angiogenesis.

Phenotypic changes which are observable with the naked eye are of special interest. Changes in the ability of the cells to grow in an anchorage-independent manner, to grow in soft agar, to form foci in cell culture, and to take up selected stains are particularly appropriate phenomena for observation and comparison.

The higher producing cell line is preferably obtained by introducing a gene encoding the Protein of Interest (POI) into a host cell. The gene may be a one isolated from the genome of an organism, a cDNA prepared from an mRNA transcript isolated from an organism, or a synthetic duplicate of a naturally occurring gene. It may also have a sequence which does not occur exactly in nature, but rather corresponds to a mutation (single or multiple) of a naturally occurring sequence. No limitation is intended on the manner in which this mutated sequence is obtained. The gene is operably linked to a promoter of gene expression which is functional in the host, such that the corresponding Protein Of Interest (POI) is stably "overproduced" in the recipient cells to differing degrees. The promoter may be constitutive or inducible. By "overproduced", I mean that the POI is expressed at higher levels in the genetically manipulated cell line than in the original cell line. This allows one to generate cell lines which contain (or secrete) from as little as a few fold to more than 100-fold elevated levels of the POI relative to the control cells.

Any method may be used to introduce the gene into the host cell, including transfection with a retroviral vector, direct transfection (e.g., mediated by calcium phosphate or DEAE-dextran), and electroporation. Preferably, a retroviral vector is used.

The host cells should exhibit a readily observable phenotypic change as a result of enhanced production of the POI. Preferably, this response should be proportional to the level of production of the POI. Finally, the cells should not spontaneously manifest the desired phenotypic change. For example, 3T3 cells form foci spontaneously. Among the preferred cell lines are Rat-6 fibroblasts, C3H10T 1/2 fibroblasts, and HL60. (HL60 is a human cell line that differentiates in response to PKC activation.) 3T3 cells may be used, but with the reservation stated above.

Generally speaking, it is preferable to maximize the ratio of production by the "overproducing" cell line to production by the "native" line. This is facilitated by selecting a host cell line which produces little or no POI, and introducing multiple gene copies and/or using high signal strength promoters.

The Rat 6 embryo fibroblast cell line is a variant of the rat embryo fibroblast cell line established by Freeman et. al., (1972) and isolated by Hsiao et al., 1986. This cell line has an unusually flat morphology, even when maintained in culture at post-confluence for extended periods of time, displays anchorage dependent growth and, thus far, has not undergone spontaneous transformation. It was also ideal for these studies since it has a very low level of endogenous PKC activity and a low level of high affinity receptors for phorbol esters.

While my most preferred host cell line is the Rat-6 fibroblast cell line, I have tested this Method with other cell types, including the mouse NIH-3T3 cell line as well as the C3H 10T1/2 cell line. Tables (a) and (b) below show the representative specific activities of seven NIH-3T3 and six C3H-10T1/2 cell lines stably overproducing PKC. I have also recently performed the same experiments with the human HeLa cell line. In each case the resulting cell lines all exhibited growth properties qualitatively identical to those described for the PKC-expressing Rat-6 fibroblast cell lines. More specifically, C3H 10T1/2 and NIH 3T3 cells which overproduced PKC exhibited increased cell density, refractility, and rounding relative to the "native" lines. The PKC-overproducing NIH 3T3 cells also displayed increased foci formation. PKC-overproducing C3H 10T1/2 cells also formed foci, but only when exposed to a phorbol ester, i.e., a PKC activator. These results clearly demonstrate that many different types of cells can be employed in this method. The experimental procedures used to generate these cell lines were also identical to those used in connection with the Rat-6 cell line.

If a cell line otherwise suitable for use as a control cell line produces excessive POI, it is possible to inhibit this production by incorporation a known inhibitor into the culture medium for both the control and test cell lines, thus achieving a more favorable ratio of production. Contrawise, if the level of POI production by the test cell line is too low, a known activator may be incorporated into the culture media.

It is desirable, but not necessary, that a suspected inhibitor or activator be tested on both a control line and an overproducing line in parallel.

What we are looking for is a increase in the phenotypic change exhibited by the cell which becomes greater with increasing expression of the POI. We call this a "graded cellular response," and it is by this specialized response that we distinguish inhibitors or activators of the POI from agents that act upon other cell metabolites to effect a phenotypic change.

Thus, in a preferred embodiment, the cell lines are assayed for their relative levels of the POI, and their ability to grow in anchorage-independent systems (e.g., matrices such as soft agar or methocel), to form small "foci" (areas of dense groups of cells clustered together and growing on top of one another) in tissue culture dishes, to take up selected stains, or to bind an appropriately labeled antibody or other receptor for a cell surface epitope. In addition to exhibiting these growth control abnormalities, such cell lines will also be sensitive in their growth properties to chemical agents which are capable of binding to, or modifying the biological effects of, the POI.

The method is particularly unique in that it can be employed to search rapidly for EITHER activators OR inhibitors of a given POI, depending upon the need. The term "activators," as used herein, includes both substances necessary for the POI to become active in the first place, and substance which merely accentuate its activity. The term "inhibitors" includes both substance which reduce the activity of the POI and these which nullify it altogether. When a POI has more than one possible activity, the inhibitor or activator may modulate any or all of its activities.

The use of this screening method to identify inhibitors or activators of enzymes is of special interest. In particular, I am interested in using it to identify inhibitors or activators of enzymes involved in tumorigenesis and related phenomena, for example, protein kinase C, ornithine decarboxylase, cyclic AMP-dependent protein kinase, the protein kinase domains of the insulin and EGF receptors, and the enzyme products of various cellular onc genes such as the c-src (P^{60src}) or c-ras (P^{20ras}) genes.

The present invention may be used to identify activators or inhibitors of receptor proteins, both cytoplasmic receptors and integral membrane receptors, provided that overproduc-

tion of the receptor protein has a detectable effect on the producing cells.

Protein kinase c (PKC) is a Ca^{2+}- and phospholipid-dependent serine/threonine protein kinase of fundamental importance in cellular growth control. PKC is activated endogenously by a wide variety of growth factors, hormones, and neurotransmitters, and has been shown to be a high affinity receptor for the phorbol ester tumor promoters as well as other agents which possess tumor promoting activity (for reviews see Nishizuka 1986; 1984; Ashendel, 1984). PKC has been shown to phosphorylate several intracellular protein substrates, including the epidermal growth factor (EGF) receptor (Hunter et al., 1984), pp60src (Gould et al., 1985), the insulin receptor (Bollag et al., 1986), p21ras (Jeng et al., 1987), and many others (Nishizuka, 1986). Several laboratories have recently isolated cDNA clones encoding distinct forms of PKC, thus demonstrating that PKC is encoded by a multigene family (Ono et al., 1986, Knopf et al., 1986, Parker et al., 1986; Coussens et al., 1986; Makowske et al., 1986; Ohno et al., 1987; Housey et al., 1987). The multiple forms of PKC exhibit considerable tissue specificity (Knopf, et. al., 1986; Brandt et al., 1987; Ohno, et al, 1987; Housey, et. al., 1987) which suggests that there may be subtle differences in the function(s) of each of the distinct forms. However, all of the cDNA clones which have been isolated thus far that encode distinct forms of PKC share at least 65% overall deduced homology at the amino acid level, and transient expression experiments with some of these cDNA clones have shown that they encode serine/threonine protein kinase activities which bind to, or are activated by, the phorbol ester tumor promoters (Knopf, et. al., 1986, Ono, et. al., 1987).

We used the PKCbeta1 cDNA clone for the present studies for the following reasons. With the exception of the brain, where its expression is very high, PKCbeta1 is expressed at very low levels in most tissues, and its expression is virtually undetectable in Rat 6 fibroblasts (see below). Thus, we reasoned that using this form would maximize the phenotypic differences observed between control cells and cells overexpressing the introduced form of PKC. The PKCbeta1 form is also of particular interest because within the PKC gene family its deduced carboxyl terminal domain displays the highest overall homology to the catalytic subunit of the cyclic AMP-dependent protein kinase (PKAc) and the cyclic GMP-dependent protein kinase (PKG) (Housey et al., 1987). The latter observation suggests that PKAc, PKG, and the beta1 form of PKC may share a common ancestral serine/threonine protein kinase progenitor, and that the additional PKC genes may have been derived through evolutionary divergence from the beta1 form.

Agents which interact with certain structural proteins, such as actin and myosin, are also of interest. Mutations in the genes expressing these proteins may be involved in tumorigenesis and metastasization. Such interactions can lead to changes in cell phenotype which can be assayed by this method.

As is set forth in greater detail below, I have produced cell lines which overproduce protein kinase C (PKC). These cell lines, unlike the control cells, grow in soft agar even in the absence of the tumor promoting phorbol ester 12-o-tetradecanoylphorbol-13-acetate (TPA). TPA has been shown to be a potent activator of PKC. When TPA is added to the growth medium the PKC-overproducing cell lines grow even better and form considerably larger colonies in soft agar. Furthermore, I have also tested known inhibitors of PKC activity which, as predicted, caused the PKC-overproducing cells to grow less well (or not at all) in soft agar. Thus, the direct utility of this method in identifying both activators or inhibitors of a gene product, in this case PKC, has been clearly demonstrated.

In additional studies with other genes, most notably the c-H-ras oncogene, the c-myc oncogene, and certain cDNA clones encoding phorbol-ester inducible proteins, overproducing cell lines which exhibited morphological changes relative to the native lines were likewise obtained, and it is believed that such cell lines may be used in screening for inhibitors and activators of the overproduced proteins. Thus it is clear that the method can be generalized to a wide variety of genes encoding proteins which are involved in cellular growth control in numerous cell types. These studies are described in more detail below.

First I tested the capability of pMV7-based expression vectors (pMV7 is my preferred transfer vector) to produce several different types of proteins in various cell lines. I used the cDNA sequences encoding the following proteins: hypoxanthine/guanine phosphoribosyltransferase (HGPRT), the human T4 lymphocyte cell surface antigen, the human T8 lymphocyte cell surface antigen, and ornithine decarboxylase (ODC). In each case the pMV7 vector was capable of producing high levels of expression of the relevant gene thereby resulting in overproduction of the corresponding protein product.

Once I had verified that the pMV7-derived expression vector could reproducibly generate cell lines which stably overproduced proteins I then tested additional genes which encode proteins, other than PKC, which are also involved in cellular growth control.

In further experiments, I have tested the method using an activated c-H-ras oncogene (T24), again in analogous fashion to the techniques described herein, and again with analogous results to those described herein for the PKC cDNA clone. Thus, the Method can also be used for the rapid development of a p21 ras inhibitor. Taken together, the results described in this application demonstrate directly that the Method described herein is clearly generalizable to any gene which is involved in any way in cellular growth control.

In further studies, we have generated additional cell lines which stably overproduce other proteins-of-interest (POIs). In one set of studies, we have introduced the beta1 form of protein kinase C (PKC) into a bone-marrow derived cell line, FDC-P1, and demonstrated that the resulting cell lines are profoundly affected by PKC activators. In a second set of studies, we have developed a screening assay system for insulin agonists and antagonists which utilizes cell lines which stably overproduce the insulin receptor. In these additional studies we have utilized different cell lines, expression vector systems, cDNAs encoding other POIs, and in general varied all of the major parameters of our initial studies in order to demonstrate the true generalizability of the system to any type of cell and any protein-of-interest.

The preferred protein inhibitor/activator drug screening method of the present invention comprises the following steps:

1. Construction of an expression vector which is capable of expressing the protein of interest in the selected host by inserting a gene encoding that protein into a transfer vector. The gene may be inserted 3' of a promoter already borne by the transfer vector, or a gene and a promoter may be inserted sequentially or simultaneously.
2. Introduction of the expression vector (a) into cells which produce recombinant retrovirus particles, or (b)

directly into host cells which will be used for subsequent drug screening tests (the resulting cells are called herein "test" cells).

In parallel, the transfer vector (i.e., the vector lacking the gene of interest and possibly a linked promoter but otherwise identical to the expression vector) is preferably also introduced into the host cells. Cell lines derived from this latter case will be used as negative controls in the subsequent drug screening tests. Alternatively, the unmodified host cells ma) be used as controls.

If 2a was employed, after an appropriate time (usually 48 hours), media containing recombinant virus particles is transferred onto host cells so as to obtain test or control cells.

3. The test and control cells are transferred to selective growth medium containing the appropriate drug which will only allow those cells harboring the expression vector containing the selectable marker gene (as well as the gene or cDNA of experimental interest) to grow. After an appropriate selection time (usually 7–10 days), individual clones of cells (derivative cell lines) are isolated and propagated separately.

4. Each independent cell line is tested for the level of production of the POI. By this method, a range of cell lines is generated which overproduce from a few fold to more that 100-fold levels of the POI. In parallel, the control cell lines which contain only the transfer vector alone (with the selectable marker gene) are also assayed for their endogenous levels of the POI.

5. Each independent line is then tested for its growth capability in soft agar (or methocel, or any other similar matrix) of various percentages and containing different types of growth media until cell lines are identified which possess the desired growth characteristics as compared to the control cell lines.

6. Each cell line is also tested for its ability to form "foci", or areas of dense cellular growth, in tissue culture plates using media containing various percentages and types of serum (20%, 10%, 5% serum, fetal calf serum, calf serum, horse serum, etc.) and under various conditions of growth (e.g. addition of other hormones, growth factors, or other growth supplements to the medium, temperature and humidity variations, etc.). In these tests, the cells are maintained at post-confluence for extended periods of time (from two to eight weeks) with media changes every three days or as required. Such growth parameters are varied until cell lines are identified which possess the desired foci formation capacity relative to the control cell lines under the identical conditions.

7. After a cell line possessing the required growth characteristics is identified, the cells are grown under the conditions determined in (5) above with the growth medium supplemented with either crude or purified substances which may contain biologically active agents specific to the POI. Thus, crude or purified substances possessing the latter properties can be rapidly identified by their ability to differentially alter the growth properties of the experimental cells (which overproduce the POI) relative to the control cells (which do not). This can be done rapidly even by simple observation with the naked eye, since the colonies which grow in soft agar after 2 weeks are easily seen even without staining, although they may be stained for easier detection.

Similarly, if the post-confluence foci formation assay is chosen, the foci which result after approximately two weeks can be easily seen with the naked eye, or these foci can also be stained. For screening very large numbers of compounds (tens of thousands or more), the entire procedure can be performed on 96 well tissue culture plates. This applies equally well for either the soft agar growth assay or the tissue culture foci formation assay. Results of the assays can be rapidly determined by measuring the relative absorbance of the test cells as compared to the control cells (at 500 nm, or another appropriate wavelength). Absorbance readings may be rapidly performed in a 96-well plate absorbance reader such as the "Titer-tek" plate reader, or any of several analogous apparatus currently available. In this fashion, thousands of compounds could be screened per month for their biological activity with very low labor and materials costs.

Furthermore, if antigen expression varies on the test cells expressing high levels of the POI relative to the control cells, a simple Enzyme-Linked Immunoadsorption Assay (ELISA) could be performed and an antibody specific to the antigen.

While the assay may be performed with one control cell line and one test cell line, it is possible to use additional lines, tests lines with differing POI levels. Also additional sets of control/test lines, originating from other hosts, may be tested.

Specific examples implementing the series of steps described above are as follows.

EXAMPLE 1

If one were interested in screening for a protein kinase C (PKC) inhibitor, cell lines would be generated and selected which grow well in soft agar (as a result of their overproduction of any form of PKC) and yet show an enhancement of their growth when compounds which are known to stimulate PKC are added to the growth medium. Appropriate control cells, of course, would not exhibit any of these characteristics. Screening for a potent PKC inhibitor could then be performed by searching for those substances which could selectively inhibit the soft agar (anchorage-independent) growth of the PKC-overproducing cell lines. Alternatively, since the PKC-overproducing cells also form small, dense foci in tissue culture, one could also screen for substances which inhibit this foci formation.

Described below are the detailed aspects of the relevant techniques and methods used to apply the principles of the invention to the problem of developing a system useful for screening for potent inhibitors of protein kinase C (PKC), a high-affinity intracellular receptor for tumor-promoting agents. The cell lines which resulted from the application of this method are highly sensitive and responsive both to agents which activate PKC as well as to those which inhibit PKC.

Construction of plasmid pRV7

The construction of pMV7 was begun with plasmid pPyori which contains the polyoma virus origin of replication cloned into the unique BamHI site of pML-1 (Lusky and Botchan, 1981). This plasmid replicates in murine cells that contain the polyoma T antigen (Dailey and Basilico, 1985). Plasmid pMV (Perkins et al., 1983) was then cleaved with HincII and BglI. The 2.29 kb fragment that contains the Moloney Leukemia Virus Long Terminal Repeats (LTR), the packaging site, the splice donor site, and the proline tRNA binding site was isolated.

During the original construction of pMV (Perkins et al., 1983) 3.95 kb was removed from the MSV genome by cleaving with PstI. This left a PstI site situated 380 bps 3' from the tRNA binding site and 308 bps from the 5' end of

the 3' LTR (Reddy et al., 1981). Xho I linkers were added to the HincII-BglI fragment, and to pPyori after it had been cleaved with EcoRI and HindIII. The two fragments were ligated, after activation of the linkers, and a plasmid, designated pMV-3, that contained the Moloney Virus control elements was isolated.

The unique EcoRI site was removed from this vector by digesting the plasmid with EcoRI and treating the linear molecules with T4 polymerase. These molecules were recircularized and a plasmid, pMV-4, lacking the EcoRI site, was isolated. An EcoRI linker was inserted into this plasmid at the PstI site between the Moloney LTRs; the resultant plasmid was designated pMV-5.

The dominant selectable marker (neo) was added to pMV-5. The first step was isolating a 1.9 kb BamHI-SalI fragment from pIPB1. This fragment contains the Herpes Simplex virus thymidine kinase (tk) promoter region and the coding sequence for the bacterial neo gene (neomycin phosphotransferase). This fragment was blunt-ended with T4 polymerase, ClaI linkers were added and the fragment was cloned into the Cla I site 165 bps 3' to the EcoRI site in pMV-5, between the LTRs. This plasmid was designated pMV5-tk neo. The tk promoter has an EcoRI site 70 bps 5' to the start of transcription. This EcoRI site was removed by partially digesting pMV5-tkneo with EcoRI, isolating the linear full length cut species, filling in the ends with T4 polymerase and recircularizing the molecule. A plasmid was chosen in which the EcoRI site previously present in the tk promoter sequence was removed, but the EcoRI site 537 bps 3' to the start of transcription of the 5' LTR was retained. This plasmid was designated pMV7 and a map of this plasmid is shown in FIG. 1B.

This vector consists of the 5' and 3' Moloney Murine Leukemia Virus (MoMuLV) LTRs, the MoMuLV RNA packaging site 3' to the 5' LTR, an Eco RI cloning site, a modified Herpes Simplex Virus thymidine kinase (tk) promoter (lacking an Eco RI site at -79 bp), and the selectable marker gene neo. cDNA clones inserted into the EcoRI cloning site are under the transcriptional control of the 5' LTR, whereas the neo gene is independently transcribed by the tk promoter. This structure favors maintenance of the functional integrity of the selectable marker without interfering with the expression of the 5' (unselected) cDNA sequence. It is known (Maddon et al., 1986; Daley et al., 1987) that when various cDNA sequences are inserted into the EcoR1 site of pMV7 they can be readily transferred into recipient cells by virus-mediated passage, are stably expressed, and yield high-level production of the corresponding protein.

Nucleotide Sequencing and Expression Vector Construction

Nucleotide sequencing of the PKC cDNA clone RP58, a full-length clone isolated from a rat brain cDNA library, which corresponds to the previously reported clone RP41, was performed as previously described (Housey et al., 1987). The full-length cDNA sequence of RP58, which encodes PKCbeta1 (FIG. 1A), was subcloned into the EcoRI site of plasmid pMV7 using standard methodology (Maniatis et al., 1983). The general structure of pMV7 is shown in FIG. 1B. The construct resulting from insertion of the PKCbeta1 gene is designated pMV7-PKCbeta1.

Isolation of cell lines stably overexpressing PKC

20 ug of CsCl banded pMV7 or pMV7-PKCbeta1 plasmid DNA were transfected (Graham and van der Eb, 1973; as modified by Wigler et al., 1977) onto subconfluent "Psi-2" cells (Mann et al., 1985). After 48 hrs the culture medium was collected, filtered through a 0.45 um filter and stored at -70° C. Recipient subconfluent Rat-6 fibroblasts (5×10^5 per 10 cm plate) were infected with the virus-containing medium in 2 ug/ml polybrene for 48 hrs. The cells, grown to confluence, were then trypsinized and replaced in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (CS) (Flow Laboratories) with 200 ug/ml of the neomycin derivative G418 (Geneticin). Resistant colonies were cloned by ring isolation after 1 week of G418 selection.

After approximately ten days of growth in selective medium, ten individual G418-resistant clones were isolated and maintained independently in G418-containing medium. These lines were designated R6-PKCbeta11 through R6-PKCbeta110 (abbreviated as R6-PKC1 through R6-PKC10). In parallel, a set of control Rat 6 lines was generated by transfection of the plasmid pMV7 (lacking the PKC cDNA insert) onto W-2 cells, infection of recipient Rat-6 cells, and selection for G418 resistance as described above for plasmid pMV7-PKCbeta1. Similarly, after ten days of growth in the G418-containing medium, five individual, well-isolated G418-resistant clones were then isolated and maintained independently. These control lines were designated R6-C1 through R6-C5.

RNA Isolation and Blot Hybridization Analyses

Poly A+ RNA isolations, gel electrophoresis, and blot hybridization analyses were performed as previously described (Housey, et. al., 1985). RNA molecular weight markers were obtained from Bethesda Research Laboratories. The 2.7kb cDNA insert of RP58 (see above) was subcloned into plasmid pKS(+) (Stratagene Cloning Systems) to yield a plasmid designated pS2-RP58. A ^{32}P-labelled probe was prepared from pS2-RP58 and used under high-stringency hybridization conditions as previously described (Housey et al., 1987).

Purification and Assay of PKC Activity From Tissue Culture Cells

The total PKC activity (membrane-associated plus cytosolic) present in cultured cells was determined after partial purification of cellular extracts as follows.

Three 10 cm plates of confluent cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS) and then 10 ml of homogenization buffer (20 mM Tris, pH 7.5, 5 mM EGTA, 5mM EDTA, 15 mM 2-mercaptoethanol, 10 ug/ml soybean trypsin inhibitor, 10 ug/ml leupeptin, 40 ug/ml phenylmethylsulfonyl fluoride), containing 0.1% Triton X-100 were added. The cells were then scraped from each of the plates, pooled and disrupted with 25 strokes in a Dounce homogenizer. The homogenate was transferred to a 15 ml disposable polystyrene tube, centrifuged at 2000×g for 5 minutes at 4° C., and the supernatant was loaded onto a 1 ml DEAE Sephacel column previously equilibrated with 10 ml homogenization buffer, at 4° C. The column was washed with 10 ml homogenization buffer and then the bound enzyme was eluted with 3 ml of homogenization buffer containing 0.5 M NaCl. Total protein concentrations were determined by the method of Bradford (1976).

The PKC activity present in the above-described partially purified cell extracts above was assayed immediately after isolation. The synthetic peptide R—K—R—T—L—R—R—L, corresponding to amino acids 651-658 of the epidermal growth factor receptor (Ullrich et al., 1984), was synthesized on an Applied Biosystems model 430A peptide synthesizer, purified by high-performance liquid chromatography, lyophilized, and stored at −20° C. The Threonine at position 654 is an in vivo substrate for PKC (Hunter, et al., 1984; Davis and Czech, 1985). This synthetic peptide is a highly specific substrate for PKC activity in vitro. (Watson et al., 1987; Woodgett, et al., 1986).

The purified material was then redissolved in sterile water at a final concentration of 100 uM and used as the phosphoacceptor substrate in the PKC assays. The general method of assay has been published in detail elsewhere (O'Brian et al., 1985). In most cases, 100 uM synthetic peptide was substituted for 2 mg/ml histone III-S as the phosphoacceptor substrate.

Cell extracts were prepared 48 hours after the cells had reached confluence. As shown in Table 1(a), eight of the ten cell lines generated by infection with the pMV7-PKCbeta1 construct (lines R6-PKC1, R6-PKC3, and R6-PKC5 through R6-PKC10) contained marked increases in PKC activity when compared to the control lines (R6-C1, -C2, and -C3). It is remarkable that cell line R6-PKC3 contained a 53 fold higher level of PKC activity than that present in the control cells. Two of the lines (R6-PKC2 and R6-PKC4), however, did not display a significant increase in PKC activity, yet they presumably had integrated the pMV7-PKC construct as evidenced by their continued G418 resistance. Subsequent studies verified that these two cell lines contained deletions in the cDNA clone encoding PKCbeta1.

In additional studies I found that the very high PKC activity in extracts of R6-PKC3 seen in the presence of 1 mM Ca^{2+} and phosphatidylserine was also apparent in the presence of 1 mM EGTA, 100 ng/ml TPA and phosphatidylserine. This very high activity was also seen when we employed histone III-S, rather than the above-described synthetic peptide, as the substrate for phosphorylation. Furthermore, even after 24 weeks of continuous growth and serial passage, the cell lines R6-PKC1 through R6-PKC6 displayed essentially the same levels of PKC activity shown in Table 1.

Autophosphorylation and Gel Electrophoresis of PKC

Cell extracts purified as described above were incubated under conditions which favor the autophosphorylation of PKC, as follows. One hundred ng of partially purified protein extract was incubated in a reaction mixture containing 80 ug/ml phosphatidylserine, 1 mM $CaCl_2$ (or 1 mM EGTA and 100 ng/ml TPA), 5 mM $MgCl_2$, and 30 uM ATP containing 100 uCi [gamma-^{32}P]ATP (New England Nuclear, NEG035). The purity of the radioactive ATP is critical to obtain reproducible autophosphorylation of PKC.

Under these conditions, it has been previously shown that PKC undergoes an autophosphorylation reaction which results in the phosphorylation of several sites on the intact enzyme (Walton et al., 1987; Huang et al., 1986, Woodgett and Hunter, 1986; Kikkawa, 1982).

Reactions were incubated at room temperature for 10 minutes and then stopped by the addition of SDS-PAGE loading buffer containing 2-mercaptoethanol. This material was then subjected to discontinuous SDS-PAGE by a modification of the method of Laemmli (1970). Twenty ug of total protein were loaded onto each lane. Following electrophoresis, the gels were fixed in 50% acetic acid, 10% ethanol, dried, and autoradiographed on Kodak XAR-5 film.

Autoradiographs of these gels (FIG. 2) revealed that the extracts prepared from four cell lines that had high PKC activity (R6-PKC1, -PKC3, -PKC5, and -PKC6, see Table 1(a)) displayed a prominent phosphorylated protein band which was about 75 kd in size, corresponding to the size of an autophosphorylated preparation of PKC obtained from rat brain (Huang et al., 1986; Housey et al., 1987). When examined in an immunoblot assay this 75 kd band also reacted with an antibody to the beta1 form of PKC (Jaken and Kiley, 1987). The control cell lines R6-C1, -C2 and -C3, and the cell lines R6-PKC2 and R6-PKC4, which did not have increased levels of PKC (see Table 1(a)) did not show this 75 kd phosphorylated band (FIG. 2), nor did they contain any bands which reacted with the antibody to the beta1 form of PKC. It is of interest that the samples obtained from the four cell lines that produced high levels of PKC also displayed weaker but distinct phosphorylated protein bands that were about 73, 60, and 49 kd in size, which were not seen (or only faintly detected) in the extracts from cells that did not have increased levels of PKC (FIG. 2). These bands may represent degradation fragments of the 75 kd PKC molecules, or specific cellular proteins that are phosphorylated by PKC.

The above-described phosphorylated protein bands were seen when either 1 mM Ca2+ plus phosphatidylserine or 100 ng/ml TPA plus phosphatidylserine were used as cofactors for PKC activation (compare even and odd numbered lanes in FIG. 2). When, however, extracts from cell lines producing high levels of PKC were incubated in an autophosphorylation reaction in the absence of such cofactors, the 75 kd band and the additional smaller bands described above were not detected. These results, taken together with the negative results obtained with extracts from the control cells (FIG. 2), clearly indicate that the phosphorylated bands reflect PKC activity.

Phorbol Ester (^{3}H-PDBU) Binding Assays

Since it has been shown that PKC is a high affinity intracellular receptor for the phorbol ester tumor promoters (for review, see Nishizuka, 1986), I also assayed a subset of the cell lines for 3H-PDBu binding using a previously described intact cell assay (Horowitz et al., 1981).

Cells were plated at 1×10^5 per 4 cm well on day 1, the medium was changed on day 2 and the cells then assayed on day 3. The monolayer was washed with 4 ml of DMEM (without serum), then 2 ml of DMEM containing 50 nM ^{3}H-PDBU (New England Nuclear; 8.3 Ci/mmol) was added and the plates were incubated for 1 hr at 37° C. to determine total binding. The fraction of the total binding that represented specific binding was determined by the addition of a 1000-fold excess of unlabelled PDBU (LC Services) to the 3H-PDBU stock (Horowitz et al., 1981). The plates were washed 3× with 4 ml ice-cold PBS. The cells were solubilized in 1 ml 1% SDS/10 mM DTT for 2 hrs at 37° C. The lysate was transferred to a scintillation vial and counted. Replica plates were used to determine the number of cells per plate and the specific binding data expressed as nanomoles 3H-PDBu bound/10^6 cells. Scatchard analyses were performed as previously described (Horowitz et al., 1981).

I found that the R6-PKC cell lines 1,3,5 and 6, all of which had high PKC enzyme activity, also had a marked increase in 3H-PDBu binding, whereas the cell line R6-PKC4, which did not display a significant increase in PKC activity, did not show an increase in 3H-PDBu binding when compared to the two control cell lines R6-C1 and R6-C2 (Table 1(a)). Scatchard analyses of the control cell line R6-C1 and of the R6-PKC3 cell line, performed as previously described (Horowitz et al., 1981), indicated that the number of high affinity receptors in the two cell lines was 1.6×10^5 and 1.4×10^6, respectively. The affinity constants were approximately the same in both cell lines (Kd=16 nM). Thus, under the assay conditions used, the R6-PKC3 cells contain about ten times the level of high affinity phorbol-ester binding sites as the control cells. It is apparent that the cell lines that express very high levels of PKC also have a significant increase in phorbol ester binding sites.

Assays for PKC-related RNA transcripts

In view of the above results, it was of interest to analyze the poly A+ RNA fraction of several of the cell lines described in Table 1(a) for the size and abundance of RNA transcripts containing sequences homologous to PKCbeta1.

Poly A+ RNA was separated on 1% agarose, 6%-formaldehyde gels, blotted onto nylon membranes, hybridized to a 32P-labelled DNA probe prepared from the full-length PKCbeta1 cDNA, and autoradiographed, as previously described (Housey, et al., 1986). As shown in FIG. 3, the lines that contained elevated levels of PKC activity (R6-PKC1,-PKC3,-PKC5, and -PKC6, see Table 1(a)) contained a prominent 6.6 kb RNA species which corresponds to the predicted size for a mRNA transcript that initiates in the 5' LTR and terminates in the 3' LTR of the pMV7-PKCbeta1 construct. This transcript was most abundant in the R6-PKC3 cell line (FIG. 3) which also expresses the highest level of PKC activity (Table 1).

On the other hand, lines R6-PKC2 and R6-PKC4, which showed no significant elevation of PKC activity (Table 1(a)), produced truncated mRNA's of approximately 5 kb and 4.8 kb, respectively. The abundance of the latter transcripts was much lower than that of the 6.6 kb transcripts present in the cell lines that expressed high levels of PKC. The neo+ phenotype and the lack of PKC activity in cell lines R6-PKC2 and R6-PKC4 suggest that the truncated mRNAs reflect deletions in PKC coding sequences in the integrated constructs carried by these cell lines. Indeed, genomic DNA blot hybridizations of clones R6-PKC2 and R6-PKC4 indicated that they contained deletions in the PKCbeta1 cDNA sequence. Neither in the parental Rat 6 cell line, nor in any of the experimental cell lines derived from these cells, was it possible to detect evidence of an endogenous transcript homologous to the PKCbeta1 probe (FIG. 3). Thus, in these cells, there is negligible expression of the endogenous gene encoding PKCbeta1.

Assays of Growth in Monolayer Culture and in Soft Agar

Cells were seeded at a density of 10^4/plate in a series of 60 mm plates, in 5 ml DMEM plus 10% CS. Twenty-four hours later, cells in triplicate plates were trypsinized and counted. This point was designated "day 0." The remaining cultures were then grown in the respective medium (i.e. plus or minus 100 ng/ml TPA) with fresh medium changes twice per week. Cell counts per plate were then determined on triplicate plates during the remainder of the growth curve (FIG. 3). The results obtained were analyzed for exponential doubling time and saturation density (Table 2). To assess growth in soft agar (anchorage independence), 2×10^4 cells were suspended in 2 ml of 0.3% Bacto agar (Difco Laboratories, Detroit, Mich.) In DMEM containing 20% fetal calf serum (FCS) and overlayed above a layer of 5 ml of 0.5% agar in the same medium, on 60 mm petri dishes. The cells were then overlaid with DMEM plus 20% FCS every 4 days. At the end of 30 days, colonies were stained with the vital stain 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolinium chloride hydrate (INT) (Sigma Chemical Co., St. Louis, Mo.) for 48 hours at 37° C., in an incubator with 5% C02 (Schaeffer and Friend, 1976), and the number of colonies counted under low power on an inverted phase microscope. The data are expressed as "cloning efficiency", i.e. number of colonies greater than 0.05 mm per plate X 100 divided by the number of cells originally seeded per plate.

Screening of a Known Activator of PKC

We found that cell lines which overproduce PKC display an exaggerated morphologic response to 12-0-tetradecanoyl phorbol-13-acetate (TPA) and altered growth control.

Changes in Morphology

To further characterize the phenotypic changes which occurred in the cell lines that overproduce PKCbeta1, lines R6-PKC3 and R6-PKC5, which contain 53- and 20-fold elevations of PKC activity, respectively, were first examined in detail with respect to their morphology, in comparison to the control cell line R6-C1. As shown in FIG. 4, Panel A, in the absence of TPA treatment all three cell lines showed the characteristic fusiform morphology of monolayer cultures of the normal parental Rat 6 fibroblast cell line. At 24 hours after treatment with 100 ng/ml TPA (FIG. 4, R6-C1: panel D), the control cell line displayed more elongated and dendritic cells and a criss-cross pattern, changes previously seen shortly after rodent fibroblasts are treated with TPA (Boreiko et al., 1980). These changes were, however, much more dramatic when the R6-PKC3 and R6-PKC5 cells were treated with TPA (R6-PKC3 and R6-PKC5, Panel D). This was particularly striking with the R6-PKC3 cells (which express the highest level of PKC) since they displayed very long cytoplasmic processes and numerous retractile cell bodies.

By 48 hours following exposure to TPA the morphology of the control cell line R6-C1 had returned to its normal appearance (FIG. 4, Panel E). On the other hand, the R6-PKC3 cells, and to a lesser extent the R6-PKC5 cells, continued to display an altered morphology. All of the cell lines were then exposed to a second, fresh dose of TPA (100 ng/ml) and examined 24 hours later (FIG. 4, Panel F), i.e., 72 hours after the first dose of TPA. The control cells failed to respond, in terms of morphologic change, to the second dose of TPA whereas the R6-PKC3 cells continued to display their altered morphology as well as an increase in cell density. The R6-PKC5 cells displayed only slight changes in morphology in response to the second dose of TPA.

Presumably, the very high level of constitutive production of PKC in the R6-PKC3 cell line is responsible for their exaggerated morphologic response to TPA as well as the failure of these cells to display the usual refractory response to TPA following an initial exposure. In normal cells, the latter response appears to be due to "down-regulation" of endogenous PKC activity. Since the R6-PKC5 cells have an intermediate level of PKC, it is not surprising that their morphologic responses to TPA are intermediate between those of the control cells and the R6-PKC3 cells.

Growth Curves

It was also of interest to determine the growth rates of these cells in monolayer culture. Detailed growth curves were performed on R6-C1, R6-PKC3 and R6-PKC5 cells in 10% calf serum and DMEM medium, in the absence and presence of 100 ng/ml TPA. The data obtained are summarized quantitatively in Table 2.

In the absence of TPA, the R6-C1 control cell line displayed the longest doubling time (26.4 hours) and the lowest saturation density (3.4×10^6 cells/plate); the R6-5 cell line had a shorter doubling time (24.9 hours) and a higher saturation density (4.8×10^6 cells/plate); and the R6-PKC3 cell line had the shortest doubling time (24.2 hours) and the highest saturation density (5.7×10^6 cells/plate). The presence of TPA decreased the doubling times, and also increased the saturation densities of all three cell lines, but the enhancement by TPA was particularly striking in the case of R6-PKC3 (Table 2).

When the cell lines were maintained in the presence of TPA for a longer period of time, the R6-PKC3 cells, but not the R6PKC5 or R6-C1 cells, showed a decline in cell density. The latter effect was due to the fact that when the R6-PKC3 cells were maintained at high cell density in the presence of TPA they became less adhesive and tended to detach from the plate.

Thus, even in the absence of TPA the R6-PKC3 cells, (which have the highest level of PKC), and to a lesser extent

the R6-PKC5 cells (which have an intermediate level of PKC), exhibit an enhancement of their growth properties which is even greater than that seen when the R6-C1 control cells are grown in the presence of TPA. Moreover, in the presence of TPA these differences in growth properties between the control and R6-PKC3 cells are even more striking.

Foci Formation

In additional studies, monolayer cultures were maintained at post-confluence for an extended period of time (28 days), with media changes every 3 days, in the absence of TPA. Whereas the control R6-C2 cell line remained a fairly uniform monolayer, after about 21 days the R6-PKC3 cell line developed numerous dense foci which were approximately 0.1–0.3 mm in diameter (FIG. 5). Furthermore, the R6-PKC3 cultures displayed numerous cells with a highly vacuolated cytoplasm which were scattered throughout the monolayer, but were not seen in the R6-C2 control culture. When the dense foci seen in the R6-PKC3 culture were picked and further passaged they grew like the parental R6-PKC3 cells and did not display a morphology typical of malignantly transformed cells. It may be surmised that these dense foci, and the vacuolated cells, reflect physiologic rather than genetic changes induced by the high level of PKC activity.

Growth on Soft Agar

I also assayed these cell lines for their ability to form colonies in soft agar, since with rodent cells the acquisition of anchorage-independent growth often correlates with tumorigenicity (Freedman and Shin, 1974). As shown in FIG. 6, when 2×10^4 cells were plated in 0.3% soft agar, both the R6-PKC3 and the R6-PKC5 cells formed numerous small colonies, whereas the control R6-C1 cells (and the parental Rat 6 cell line) failed to grow and persisted as single cells. In addition, when TPA (100 ng/ml) was added to the agar medium, the colony sizes and cloning efficiencies of the R6-PKC3 and RC-PKC5 cells were enhanced (FIG. 6 and Table 2), but the R6-C1 cells still failed to grow in agar.

The cloning efficiencies and colony sizes of the R6-PKC3 cultures were always greater than those of the R6-PKC5 cultures, both in the absence and presence of TPA (Table 2), presumably reflecting the higher level of PKC activity in the former cell line. Thus, it is clear that the overproduction of PKC is associated with the acquisition of anchorage-independent growth in Rat 6 cells. The sizes of the colonies formed in agar by the PKC cell lines are smaller than those formed by Rat 6 cells transformed by an activated c-H-ras oncogene, which have a diameter of about 0.5–1.0 mm.

Further Screening of Activators and Inhibitors of PKC

Additional compounds tested included the tumor promoters teleocidin, aplysiatoxin, and mezerein, all of which are known activators of PKC (O'Brian, et al., CSH, 1985). Furthermore, a known inhibitor of PKC, H-7 (Kawamoto and Hidaka, 1984), also modulated the growth of the cells in the expected manner.

Moreover, this method has been used to establish that the anti-estrogen tamoxifen (O'Brian, et al, Cancer Res., 1985), which inhibits PKC enzyme activity in a cell-free assay, is capable of completely inhibiting the growth in agar of all of the cell lines overproducing PKC. Inhibition of the growth of the R6-PKC3 cells in agar in the presence of tamoxifen provided critical evidence that tamoxifen could inhibit PKC-mediated stimulation of cellular growth.

Furthermore, the concentration of the inhibitor necessary to completely inhibit the growth of each cell line was roughly proportional to the amount of PKC being overproduced in that particular cell line. In other words, there is a direct relationship between the molar amount of inhibitor required to prevent cell growth and the molar amount of PKC present in each cell line.

In addition, I have demonstrated the analogous relationship between the molar amounts of PKC activators and the molar amounts of PKC present in cells. In other words, there is a direct relationship between the molar amount of activator required to stimulate cell growth and the molar amount of PKC present in cells. Thus, this work establishes, for the first time, the fact that stable overproduction of a protein in mammalian cells can result in a novel cellular phenotype(s) (in this case anchorage independence) which can be directly modulated by chemical agents which interact with the protein.

EXAMPLE 2

If one were interested in screening for a potent inhibitor of the c-H-ras oncogene product (the p21 protein) then one would generate cells which grow well in soft agar with appropriate media conditions when p21 is stably overproduced at a certain level, but not at all when p21 is present at wild type levels. Screening for a potent p21 inhibitor could then be performed as described in Example 1 above.

EXAMPLE 3

The same basic techniques would also apply to genes (or cDNA sequences) which have been mutated either by laboratory design (e.g. site-directed mutagenesis) or as a result of naturally occurring events. Thus, any of the known point mutations in the ras oncogene which result in greater capability by the mutated gene to transform normal cells to cancerous ones could be employed in the same basic procedures as described above.

EXAMPLE 4

The previously mentioned retroviral expression vector pMV7 was utilized to introduce a cDNA encoding the beta1 isoform of PKC into FDC-P1 cells. FDC-P1 is a bone marrow-derived cell line which requires interleukin-3 (IL-3) for growth. In these studies, the hemopoietic cell line FDC-P1 was utilized in order to demonstrate the utility of the screening method even in cells which have very stringent growth requirements and would otherwise be very difficult to maintain in culture.

The following abbreviations are used herein: SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis (beta aminoethyl ether) N,N,N',N'-tetraacetic acid; EDTA, ethylene diamine tetraacetic acid; TPA, 12-0-tetradecanoyl phorbol-13-acetate (also known as PMA); CHO, Chinese hamster ovary cells; DEAE, diethyl aminoethyl.

Cell Culture

FDC-P1 cells were routinely maintained in Dulbecco's modified eagle's medium containing 10% iron-supplemented calf serum. We added 10% WEHI-3B cell-contained medium as a source of IL-3 and subcultured the cells twice weekly to give 2x10E5 cells/ml.

Isolation of cell lines stably overexpressing PKC

Subconfluent [PSI]-2 cells expressing a cDNA clone encoding the complete PKCbeta1 sequence (Housey et al., 1988) and containing the G418 resistance gene were irradiated with 3000 rad. These cells were cocultivated with FDC-P1 grown as described above, and 8 ug/ml of polybrene was added. After 48 hours, the FDC-P1 cells were pelleted, plated in 35mm petri dishes containing 0.3% agar, 10% calf serum, and 10% WEHI conditioned medium, and selected with 0.3 mg/ml of G418. After 10 days colonies were aspirated from the agar and grown in liquid medium.

RNA isolation and blot hybridization

Total RNA was extracted (Chomczynski, 1987) from FDC-P1 cells and run on a 1.5% agarose gel, blotted to nitrocellulose, and probed with the nick-translated EcoRI-PstI fragment of the RP-58 cDNA encoding PKCbeta1 (Housey, 1988). In separate experiments nitrocellulose blots were probed with either the BamHI fragment of the murine GM-CSF cDNA (Lee, 1985) or the XhoI fragment of the murine IL-3 gene (Yokota, 1984), clones donated by Dr. Lee and labelled by nick-translation to 1×10E8 cpm/ug. The labelled PstI fragment of tubulin was used to examine RNA loading.

Western blotting of PKC

FDC-P1 cells were placed directly into a buffer containing 1% SDS; 2 mM EGTA; 2nM EDTA; 20 mM Tris-HCl, pH 8.0, and 1% beta mercaptoethanol. The sample was boiled and pelleted for 30 min. at 20 degrees C at 200,000×g. Then 80 ug of supernatant protein was electrophoresed on a 10% SDS polyacrylamide gel, and the proteins were transferred to nitrocellulose. The blot was blocked with bovine serum albumen and was probed with an antibody made to a conserved region of PKC (LLNQEEGEYYNVPIPE) which recognizes all forms of the enzyme (Stabel, 1987). The PKC standard was purified from rat brain by previously published procedures (Kraft, 1988).

Stable Overproduction of PKCbeta1 in FDC-P1 Cells

In order to generate FDC-P1 cells which stably overproduced PKCbeta1, we cocultured FDC-P1 cells, which are dependent upon a source of IL-3 for continued growth, with [PSI]-2 cells which produce a retrovirus encoding the complete PKC beta1 cDNA clone in the pMV7 expression vector. After 48 hours of cocultivation, the FDC-P1 cells were plated in soft agar in medium containing G418 and IL-3, and after 10 days of selection several colonies were aspirated and grown in liquid culture.

Approximately 30 of these clones were screened by western blotting with a PKC antibody which recognizes all forms of PKC. A number of clones demonstrated elevated levels of PKC beta1 protein by Western blot analysis. Northern blot analyses were consistent with the western blot results and demonstrated that clones 40, 43, and 51 exhibited hybridizable bands to the EcoRI-PstI fragment of the PKC-beta1 clone, whereas the parental cell line and clone 2 (which lacked any PKCbeta 1-specific mRNA), were negative. Furthermore, the total PKC activity in these cells was examined using histone IIIs as a phosphotransferase acceptor (Table 4). As expected, Clone 2 and the parental cell line had approximately the same PKC activity, whereas clones 40, 43, and 51 demonstrated approximately 20-fold increases in activity.

PKC Activators Induce Significant Morphologic Changes in FDC-P1 Cells That Stably Overproduce PKC

We then tested the morphologic responsiveness of the FDC-P1 cells to activators of PKC induce profound morphologic changes in the FDC-P1 cells. Several cell lines were plated at 3×10E4 cells/ml and treated with 100 nM TPA. After 12 hours, representative fields were photographed. The PKC beta1 overproducing cells exhibited profound morphologic changes, including a generalized rounding up of the cells followed by the rapid formation of dense, cellular clumping. The latter clumps are easily visualized with standard cell stains such as Giemsa, and stain much darker than the corresponding control cells under these conditions.

Screening Assay to Detect Activators and Inhibitors of PKC

Thus, as with our previous findings for other cell lines such as NIH 3T3, Rat 6, and C3H 10T 1/2, this system also provides a rapid, powerful cell-based assay for the discovery and characterization of both activators and inhibitors of PKC as follows. Compounds could be rapidly screened for new activators of PKC by testing their ability to induce cellular clumping in the Test cells, while leaving the Control cells essentially unaffected. In addition, if one wanted to screen for PKC inhibitors, compounds could be rapidly screened for their ability to inhibit the effects of PKC by testing their ability to prevent cellular clumping induced by TPA in the Test cells, while again leaving the Control cells essentially unaffected. In this latter test, Control and Test cells would be incubated simultaneously with 100 nM TPA and the individual compounds to be tested. After 12 hours the Control and Test cells would be stained and scored for the inhibition of cellular clumping in the Test cells while leaving the Control cells unaffected.

EXAMPLE 5

In further studies designed to test the utility of this system with other proteins of interest (POI), we have utilized the human insulin receptor as a model POI. Cell lines which stably overproduce the insulin receptor were used to develop an assay system which is capable of detecting both agonists and antagonists of insulin action.

In the majority of vertebrates, insulin is the primary hormone involved in the homeostatic control of blood glucose levels. The many effects of insulin action on cells include stimulation of glucose, protein, and lipid metabolism as well as effects on RNA and DNA synthesis (Metcalf, 1985). These actions of insulin on cells begin at the molecular level through the binding of insulin to the insulin receptor. The insulin receptor is present on virtually all mammalian cells, but its number varies from as few as 40 to more than 200,000 receptors per cell (Kahn and White, 1988, and references therein). The insulin receptor is a heterotetrameric glycoprotein consistent of two alpha and two beta subunits. The alpha subunits, Mr=135,000, are located entirely extracellularly, whereas the beta subunits are transmembrane proteins which possess intrinsic tyrosine kinase activity. The alpha and beta subunits are attached by disulfide bonds on the extracellular side of the cell membrane. Both the alpha and the beta chains of the mature receptor molecule are derived from a single chain precursor polypeptide (the "proreceptor") which in turn is encoded by a single gene.

It is now known that the beta subunit of the insulin receptor is a protein tyrosine kinase which phosphorylates substrate proteins on tyrosine residues. This tyrosine kinase activity of the beta subunit is stimulated by the binding of insulin to the alpha subunit, and it has been shown that the tyrosine kinase activity of the beta subunit is essential for insulin action on target cells (Kahn and White, 1988, and references therein).

Generation of CHO Cell Lines Stably Overproducing the Human Insulin Receptor

In additional demonstrations of the utility of the method, we have employed Chinese Hamster Ovary (CHO) cell lines which stably overproduce the human insulin receptor (HIR) (White et al., 1988). These cell lines were generated using approaches analogous to those previously described for the PKC-overproducing cell lines. However, in these HIR studies we utilized a different expression vector, distinct cell lines, and an alternative method of gene transfer (direct transfection as opposed to virus-mediated transduction as used in the PKC experiments previously described). Briefly, CHO cells were co-transfected with the transfer vector pSVEneo (containing the neomycin resistance gene) and the

expression vector pCVSVHIRc as previously described (White et al., 1988). After twenty four hours, the transfected cells were placed into 800 ug/ml G418 to select resistant cells. Approximately two weeks later, G418-resistant clones were isolated and subcloned. Clonal cell lines which express high levels of surface insulin receptors were selected by fluorescence-activated cell sorting. One such line (CHO/HIRC), which stably overproduced approximately 80,000 insulin receptors per CHO cell, was utilized in these experiments. A control CHO cell line (CHO/NEO), harboring only the pSVEneo transfer vector, were generated in analogous fashion. Detailed preliminary characterizations of this cell line have been described (White et al., 1988).

Morphologic and Growth Control Effects of Insulin Treatment on CHO Cells Stably Overproducing the Human Insulin Receptor

We then tested the ability of the CHO/HIRC and CHO/NEO cells to respond to the following insulin treatment protocol. Control cells, hereinafter termed CHO/NEO (which harbor pSVEneo alone), and the Test cells, termed CHO/HIRC (which stably overproduced about 80,000 insulin receptors per cell), were incubated in the presence and absence of insulin. Control (CHO/NEO) and Test (CHO/HIRC) cell lines were exposed to 400 nM of insulin and the resultant morphologic changes were observed and photographed using a Bausch and Lomb BL-200 phase-contrast microscope with a Nikon FM-200 camera attached.

After 18 hours the test cells began to exhibit profound morphologic changes, including increased refractility, cellular rounding, and decreased adherence to the culture plate (anchorage dependence). These effects were further pronounced at 24 hours after exposure. Indeed, by 48 hours after treatment, virtually all of the test cells had completely detached from the plate, whereas the control cells were still largely intact. If the test cells were transferred to new plates in medium lacking high levels of insulin, however, they were completely viable and would attach to the plate and continue to grow normally. Thus, although insulin treatment could rapidly induce the anchorage independent phenotype, the cells remained completely viable for future growth if insulin was subsequently removed from their growth medium.

Screening Assay to Detect Activators and Inhibitors of the Insulin Receptor

Thus, in yet another demonstration of the utility of our method, the system described above provides a rapid and powerful screening method for the discovery and characterization of insulin agonists and antagonists as follows.

Compounds may be screened by testing their ability to preferentially induce anchorage-independent growth in the Test cells relative to the Control cells. Each compound would be applied to both the Control and the Test cells growing in multi-well plates, and 48 hours later the wells would be stained with a simple dye such as Giemsa (Housey et al., 1988 and references therein).

Whereas the Control CHO/NEO cells would consistently remain attached to the plate and stain strongly with Giemsa, the Test CHO/HIRC cells would be induced to detach from the plate by compounds which mimic the effects of insulin and therefore would no longer be stained by Giemsa. A standard microtitre well plate-reader (such as those produced by SLT America, Inc.) could easily read the absorbance of each well due to the staining procedure(s) used.

Thus, in this manner thousands of compounds could be screened for their ability to mimic the effects of insulin. Such agents are, of course, insulin agonists. However, as with the methods described above for PKC, the method could also be utilized for the discovery of insulin antagonists by simply treating the Control and Test Cells with potential antagonist compounds in the presence of insulin.

By this latter approach, compounds capable of inhibiting the effects of insulin action would prevent the induction of anchorage independence in the CHO/HIRC cells, whereas compounds with no effect would be unable to prevent the induction of anchorage-independent growth in the Test Cells. Most importantly, this insulin receptor assay system demonstrates yet another direct application of the utility of stable overproduction of a protein-of-interest (POI) for the development of powerful assay systems capable of detecting activators or inhibitors of any POI.

Julius, et al., Science, 244: 1057-62 (Jun. 2, 1989) reports the production of a cell line which overproduces the serotonin receptor 5HT1c by cloning its cDNA into the expression vector pMV7. The transformed cell lines exhibited a change in morphology, specifically, the formation of foci. Mesulergine, a serotonin antagonist, completely blocked the formation of foci. This paper was published after the filing date of the parent application, Feb. 10, 1988, and is therefore cited merely to further demonstrate the broad applicability of the screening method described herein.

Further Modifications

Use of any expression vector capable of stably overexpressing a given gene in a recipient cell could be used with success in the procedures described herein. The retroviral vector which I used here was particularly well suited to the problem since I had designed it specifically for these purposes. However, other similar vector systems would work. Also, one could do co-transfection of an experimental gene inserted in one plasmid vector along with a second plasmid containing the selectable marker gene (rather than having both the experimental gene and the selectable marker gene on the same plasmid vector). This is more difficult and less efficient than using the pMV7 vector, but it would work to some extent.

Any growth medium, in addition to soft agar or methocel, which tends to prohibit the growth of normal, non-transformed cells, could also be used.

A culture of *E. coli* DH1 bearing the plasmid denoted pMV7-RP58 (pMV7-PKC beta1), was deposited under the Budapest Treaty with the American Type Culture Collection on Feb. 11, 1988, ATCC No. 67654. The deposit of this plasmid is not to be construed as an admission that the deposit is required for enablement or that the disclosure is limited to the deposited vector or gene.

TABLE 1(a)

PKC Activity and Phorbol Ester Binding in Rat 6 Cells Infected with pMV7 or pMV7-PKCbeta1 Constructs

Cell Line	PKC Activity Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control	^{3}H-PDBU Binding (pmol/10^6 cells)
R6-C1	100	—	1.6
R6-C2	85	—	1.3
R6-C3	150	—	1.5
mean + s.d.	100 + 34	1	
R6-PKC1	2480	23	12.7
R6-PKC2	85	1	ND
R6-PKC3	5840	53	9.9
R6-PKC4	190	2	0.7
RE-PKC5	2200	20	5.8
R6-PKC6	4600	42	7.1
R6-PKC7	2150	20	ND
R6-PKC8	3280	30	ND

TABLE 1(a)-continued

PKC Activity and Phorbol Ester Binding in Rat 6 Cells Infected with pMV7 or pMV7-PKCbetaI Constructs

Cell Line	PKC Activity Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control	3H-PDBU Binding (pmol/10^6 cells)
R6-PKC9	4990	45	ND
R6-PKC10	5050	46	ND

TABLE 1(b)

PKC Activity in NIH-3T3 Cell Lines Infected with pMV7 or pMV7-PKCbetaI Constructs

NIH-3T3 Cell lines Increase (Controls)	PKC Specific Activity (pmol/min/mg prot)	Fold Relative to Control
3T3-C1	110	—
3T3-C2	150	—
3T3-C3	90	—
mean +/- s.d. (control lines)	115 +/- 30	
NIH-3T3 PKC-Overproducing Cell Lines		
3T3-PKC1	2570	22
3T3-PKC2	3640	32
3T3-PKC3	1960	17
3T3-PKC4	1240	11
3T3-PKC5	4190	36
3T3-PKC6	2110	18
3T3-PKC7	5050	44

TABLE 1(c)

PKC Activity in C3H-10T1/2 Cell Lines Infected with pMV7 or pMV7-PKCbetaI Constructs

C3H-10T1/2 Cell lines Increase (Controls)	PKC Specific Activity (pmol/min/mg prot)	Fold Relative to Control
C3H-C1	115	—
C3H-C2	155	—
C3H-C3	130	—
C3H-C4	185	—
mean +/- s.d.	145 +/- 30	
C3H-10T1/2 PKC-Overproducing Cell Lines		
C3H-PKC1	2340	16
C3H-PKC2	5010	35
C3H-PKC3	950	7
C3H-PKC4	1360	9
C3H-PKC5	4340	30
C3H-PKC6	7460	51

Legend to Table 1

Control cell lines were obtained by infecting rat-6-fibroblast, NIH-3T3 and C3H-10T1/2 cells with the pMV7 vector itself (lacking the PKC cDNA insert) whereas the PKC-overproducing cell lines were obtained from rat-6-fibroblast, NIH-3T3 and C3H-10T1/2 cells infected with the pMV7-PKCbeta construct, as described above. Total PKC activity was partially purified from each of the cell lines and assayed in the presence of 1 mM Ca2+ and 80 ng/ml phosphatidylserine, using the synthetic peptide R—K—R—T—L—R—R—L as substrate. Specific activity is reported as the amount of incorporation of 32P into the synthetic peptide substrate per milligram of protein per minute. All assays were done in duplicate and varied by less than 10%.

TABLE 2

GROWTH PROPERTIES OF RAT 6 CELL LINES OVERPRODUCING PKC AND THEIR RESPONSES TO TREATMENT

		Growth in Culture			
Cell Line	TPA Add	Monolayer Doubling Time (hrs)	Saturat. Density (× 10^6)	Agar Effic. (%)	Colony Size (mm)
R6C1	–	26.4	3.4	0	—
(control)	+	24.6	4.2	0	—
R6-PKC3	–	24.2	5.7	25.1	0.10–0.15
(test)	+	21.5	10.0	29.7	0.15–0.35
R6-PKC5	–	24.9	4.8	17.3	0.05–0.10
(test)	+	22.9	7.0	34.7	0.10–0.15

Table 2.

The cells were grown as described above. The "doubling times" relate to the initial exponential phase of cell growth and the "saturation density" represents the number of cells per 6 cm plate on day 11. The data are taken from the experiment shown in FIG. 3.

TABLE 3

Inhibition of Growth in Agar of PKC-Overproducing Cell Lines Using Various Inhibitors

Inhibitor Agar (%)	Cell Line	Conc. Inhibitor (uM)	Effic. of Growth in
H-7	R6-PKC3	0	27
H-7	R6-PKC3	2	25
H-7	R6-PKC3	5	22
H-7	R6-PKC3	10	17
H-7	R6-PKC3	50	4
H-7	R6-PKC3	100	0
H-7	R6-PKC5	0	20
H-7	R6-PKC5	2	21
H-7	R6-PKC5	5	16
H-7	R6-PKC5	10	4
H-7	R6-PKC5	50	1
H-7	R6-PKC5	100	0
Tamoxifen	R6-PKC3	0	25
Tamoxifen	R6-PKC3	5	27
Tamoxifen	R6-PKC3	20	22
Tamoxifen	R6-PKC3	50	15
Tamoxifen	R6-PKC3	100	6
Tamoxifen	R6-PKC3	200	0
Tamoxifen	R6-PKC5	0	19
Tamoxifen	R6-PKC5	5	19
Tamoxifen	R6-PKC5	20	17
Tamoxifen	R6-PKC5	50	8
Tamoxifen	R6-PKC5	100	0
Tamoxifen	R6-PKC5	200	0
Staurosporine	R6-PKC3	0	29
Staurosporine	R6-PKC3	0.001	29
Staurosporine	R6-PKC3	0.005	26
Staurosporine	R6-PKC3	0.010	15
Staurosporine	R6-PKC3	0.050	7
Staurosporine	R6-PKC3	0.250	0
Staurosporine	R6-PKC5	0	22
Staurosporine	R6-PKC5	0.001	21
Staurosporine	R6-PKC5	0.005	17
Staurosporine	R6-PKC5	0.010	10
Staurosporine	R6-PKC5	0.050	2
Staurosporine	R6-PKC5	0.250	0

TABLE 4

PKC Activity in FDC-P1 Cells Transfected with a Retrovirus Encoding PKCbeta1

Cell Line	PKC Activity (pmol/min/ug prot)	Fold Increase Relative to Parental Cell Line
Parental Control	78	1
FDC-P1 clone 2	64	1
FDC-P1 clone 4	1873	24
FDC-P1 clone 43	1558	20
FDC-P1 clone 51	1135	15

Approximately 3×10E7 cells in the logarithmic phase of growth were homogenized, supernatants places over a DEAE-cellulose, and eluates assayed as previously described (Kraft, 1983).

References

Ashendel, C. The Phorbol Ester Receptor: a phospholipid-regulated protein kinase. (1984) Biochim. Biophys. Acta 822, 219–242.

Bollag, G. E., Roth, R. A., Beaudoin, J., Mochly-Rosen, D., Koshland, D. E. Jr. (1986) Protein kinase C directly phosphorylates the insulin receptor in vitro and reduces its protein-tyrosine kinase activity. Proc. Natl. Acad Sci. USA 83, pp 5822–4.

Boreiko, C., Mondal, S., Narayan, S., and Heidelberger, C. (1980) Effect of 12–0-Tetradecanoylphorbol-13-acetate on the Morphology and Growth of C3H/10/2 Mouse Embryo Cells. Cancer Res. 40, 4709–4716.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brandt, S. J., Niedel, J. E., Bell, R. M., Young, W. S. (1987) Distinct patterns of expression of different protein kinase C mRNAs in rat tissues. Cell 49, pp 57–63.

Catino, J. J., Francher, D. M., Edinger, K. J., and Stringfellow, D. A. (1985) A microtitre cytotoxicity assay useful for the discovery of fermentation-derived antitumor agents. Cancer Chemother. Pharmacol. 15, 240–243.

Chomczynski. P., Sacchi, N. (1987), Anal. Biochem., 162: 156–159.

Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U., & Ullrich, A. (1986) Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signalling pathways. Science 233, 859–866.

Dailey, L., and Basilico, C. (1985). Sequences in the polyomavirus DNA regulatory region involved in viral DNA replication and early gene expression. J. Virol. 54, 739–749.

Daley, G. Q., McLaughlin, J., Witte, O. N., Baltimore, D. The CML-Specific P210 bcr/abl Protein, Unlike v-abl, Does Not Transform NIH/3T3 Fibroblasts Science 237, pp 532–535.

Davis, R. J., and Czech, M. P. (1985) Platelet-derived growth factor mimics phorbol diester action on epidermal growth factor receptor phosphorylation at threonine 654. Proc. Natl. Acad. Sci. 82, 4080–4084.

Freeman, A. E., Price, P. J., Igel, H. J., Young, J. C., Maryak, J. M. Huebner, R. J. (1970) Morphological transformation of rat embryo cells induced sa dimethylnitrosamine and murine leukemia viruses. J. Natl. Cancer Inst. 44, pp 65–78.

Freedman, V. H. and Shin, S. (1974) Cellular Tumorigenicity in nude Mice: Correlation with Cell Growth in Semi-Solid Medium. Cell 3, 355–359.

Graham, F. L., and van der Eb, A. J. (1973) A new technique for the assay of infectivity of human adenovirus DNA. Virology 52, 456–467.

Gould, K. L., Woodgett, J. R., Cooper, J. A., Buss, J. E., Shalloway, D., Hunter, T. (1985) Protein Kinase C Phosphorylates pp60src at a novel site. Cell 42, pp 849–857.

Horowitz, A. D., Greenebaum, E. and Weinstein, I. B. (1981) Identification of receptors for phorbol ester tumor promoters in intact mammalian cells and of an inhibitor of receptor binding in biologic fluids. Proc. Natl. Acad. Sci. U.S.A. 78, pp 2315–2319.

Housey, G. M., O'Brian, C. A., Johnson, M. D., Kirschmeier, P., and Weinstein, I. B. (1987) Isolation of cDNA clones encoding protein kinase C: Evidence for a protein kinase C-related gene family. Proc. Natl. Acad. Sci. U.S.A. 84, pp 1065–1069.

Housey, G. M., Kirschmeier, P., Garte, S. J., Burns, F., Troll, W., & Weinstein, I. B. (1985) Expression of long terminal repeat (LTR) sequences in carcinogen-induced murine skin carcinomas. Biochem. Biophys. Res. Commun. 127, 391–398.

Hsiao, W.-L. W., T. Wu, Wainstein, I. B., (198C) Oncogene-Induced Transformation of a Rat Embryo Fibroblast Cell Line is Enhanced by Tumor Promoters. Mol. Cell. Biol. 6, pp 1943–1950.

Hunter, T., Ling, N., Cooper, J. A. (1984) Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature 311, 480–3.

Huang, K. P., Nakabayashi, H., Huang, F. L. (1986) Isozymic forms of rat brain Ca2+-activated, phospholipid-dependent protein kinase. Proc. Natl. Acad. Sci. 83, 8535–8539.

Housey, G. M., Johnson, M. D., Hsiao, W. L., O'Brian, C. A., Murphy, J. P., Kirschmeier, P. and Weinstein, I. B., (1988), Cell, 52: 343–54.

Jaken, S. and Kiley, S. (1987) Purification and characterization of three types of protein kinase C from rabbit brain cytosol. Proc. Natl. Acad. Sci. U.S.A. 84, pp 4418–4422.

Jeng, A. Y., Srivastava, S. K., Lacal, J. C., Blumberg, P. M. (1987) Phosphorylation of ras oncogene product by protein kinase C. Biochem. Biophys. Res. Commun. 145, pp 782–8.

Johnson, M. D., Housey, G. M., Kirschmeier, P., and Weinstein, I. B. (1987) Molecular Cloning of Gene Sequences Regulated by Tumor Promoters Through Protein Kinase C. Mol. Cell Biol. 7, 2821–2829.

Kahn, C. R., White, M. F. (1988), The Insulin Receptor and the Molecular Mechanism of Insulin Action, J. Clin. Invest., 82: 1151–56.

Kajikawa, N., Kishimoto, A., Shiota, M., & Nishizuka, Y. (1983) Ca2+-dependent neutral protease and proteolytic activation of Ca2+-activated, phospholipid-dependent protein kinase. Methods. Enzymol. 102, 279–290.

Kawamoto, S. and Hidaka, H. (1984) 1-(5-Isoquinolinesulfonyl) -2-methyl-piperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. Biochem. Biophys. Res. Commun. 125, 258.

Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982) Calcium-activated, phospholipid-dependent protein kinase from rat brain. Subcellular distribution, purification, and properties. J. Biol. Chem. 257, 13341–13348.

Kirschmeier, P. T., Housey, G. M., Johnson, M. D., Perkins, A. S., and Weinstein, I. B. (1988) Construction and

Characterization of a Retroviral Vector Demonstrating Efficient Expression of Cloned cDNA Sequences. DNA, in press.

Knopf, J. L., Lee, M-H. Sultzman, L. A., Kriz, R. W., Loomis, C. R., Hewick, R. M. & Bell, R. (1986) Cloning and expression of multiple protein kinase C cDNAs. Cell 46, 491–502.

Kraft, A. S., Reeves, J. A. and Ashendel, C. L. (1988), J. Biol. Chem., 263: 8437–42.

Kraft and Anderson, W. B. (1983), J. Biol. Chem., 258: 9178–9183.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Leach, K. L., James, M. L., & Blumberg, P. M., (1983) Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol. Proc. Natl. Acad. Sci. U.S.A. 80, 4208–4212.

Lee, F., Yokota, T., Otsuka, T., Gemmell, L., Larson, N., Luh, J., Arai, K-I., and Rennick, D. (1985), Proc. Nat. Acad. Sci. U.S.A., 82: 4360–64.

Lusky, M., and Botchan, M. (1981). Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. Nature 293, 74–81.

Maddon, P. J., Dalgleish, A. G., McDougal, J. S., Clapham, P. R., Weiss, R. A., Axel, R. (1986) The T4 Gene Encodes the AIDS Virus Receptor and Is Expressed in the Immune System and the Brain. Cell 47, pp 333–348.

Makowske, M., Birnbaum, M. J., Ballester, R., Rosen, O. M. (1986) A cDNA encoding PKC identifies two species of mRNA in brain and GH3 cells. J. Biol. Chem. 261, pp 3389–13392.

Maniatis, T., Fritsch, E. F., & Sambrook, J. eds. (1983) in Molecular Cloning : A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.)

Mann, R., Mulligan, R. C., and Baltimore, D. (1983) Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell 33, 153–159.

Nishizuka, Y. (1986) Studies and Perspectives of Protein Kinase C. Science 233, 305–312. Nishizuka, Y. (1984) The Role of Protein Kinase C in Cell Surface Transduction and Tumour Promotion. Nature (London) 308, 693–698.

Metcalf, D., (1985) Science, 229, 16–22.

O'Brian, C. A., Lawrence, D. S., Kaiser, E. T., & Weinstein, I. B. (1984) Protein kinase C phosphorylates the synthetic peptide Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val in the presence of phospholipid plus either Ca2+ or a phorbol ester tumor promoter. Biochem. Biophys. Res. Commun. 124, 296–302.

O'Brian, C., Arcoleo, J., Housey, G. M., & Weinstein, I. B. (1985) in Cancer Cells 3, eds. Feramisco, J., Ozanne, B. & Stiles, C. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) 359–363.

O'Brian, C. A., Liskamp, R. M., Solomon, D. H. and Weinstein, I. B. (1985) Inhibition of Protein Kinase C by Tamoxifen. Cancer Res. 45, 2462–2465.

Ono, Y., Kurokawa, T., Fujii, T., Kawahara, K., Igarashi, K., Kikkawa, U., Ogita, K., Nishizuka, Y. (1986) Two types of complementary DNAs of rat brain protein kinase C. FEBS 206, 347–52.

Ono, Y., Kikkawa, U., Ogita, K., Tomoko, F., Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K., Nishizuka, Y. (1987) Expression and Properties of Two Types of Protein Kinase C: Alternative splicing from a Single Gene. Science 236, pp 1116–1120.

Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T., Hidaka, H. (1987) Tissue-specific expression of three distinct types of rabbit protein kinase C. Nature (London) 325, pp 161–6.

Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., & Ullrich, A. (1986) The complete primary structure of protein kinase C–the major phorbol ester receptor. Science 233, 853–858.

Perkins, A. S., Kirschmeier, P. T., Gattoni-Celli, S., and Weinstein, I. B. (1983). Design of a retrovirus-derived vector for expression and transduction of exogenous genes in mammalian cells. Mol. Cell. Biol. 3, 1123–1132.

Pontremoli, S., Melloni, E., Michetti, M., Sparatore, B., Salamino, F., Sacco, O., and Horecker, B. L. (1987) Phosphorylation and proteolytic modification of specific cytoskeletal proteins in human neutrophils stimulated by phorbol-12-myristate 13-acetate. Proc. Natl. Acad. Sci. 84, 3604–3608.

Sibley, D. R., Benovic, J. L., Caron, M. G., Lefkowitz, R. J. (1987) Regulation of transmembrane signalling by receptor phosphorylation. Cell 48, 913–922.

Stabel, S., Rodriguez-Pena, A., Young S., Rozengurt E., and Parker, P. J. (1987), J. Cell Physiol., 130: 111–117.

Uehara, Y., Hori, M., Takeuchi, T., Umezawa, H. (1985) screening of Agents Which Convert 'Transformed Morphology' of Rous Sarcoma Virus-Infected Rat Kidney Cells to 'Normal Morphology': Identification of an Active Agent as Herbimycin and its Inhibition of Intracellular src Kinase. Jpn. J. Cancer Res. 76, 672–675.

Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., and Seeburg, P. H. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309, 418–425.

Walton, G. M., Bertics, P. J., Hudson, L. G., Vedvick, T. S., Gill, G. N. (1987) A Three-Step Purification Procedure for Protein Kinase C: Characterization of the Purified Enzyme. Anal. Biochem. 161, 425–437.

Weinstein, I. B., (1987) Growth Factors, Oncogenes, and Multistage Carcinogenesis. J. Cell. Biochem. 33, pp 213–224.

White, M. F., Livingston, J. N., Backer, J. M., Lauris, V., Dull, T. J., Ullrich, A., Kahn, C. R., Mutation of the Insulin Receptor at Tyrosine 960 Inhibits Signal Transmission but Does Not Affect its Tyrosine Kinase Activity (1988), Cell, 54: 641–649.

Wigler, M., Silverstien, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C, and Axel, R. (1977). Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11, 223–232.

Woodgett, J. R., Gould, K. L., and Hunter, T. (1986) Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. Eur. J. Biochem. 161 177–184.

Yokota, T., Lee, F., Rennick, D., Hall, C., Aria, N., Mosmann, T., Nabel, G., Cantor, H., and Arai, K-I., (1984), Proc. Nat. Acad. Sci. USA 81: 1070–74.

Young, S., Parker, P. J., Ullrich, A., and Stabel, S. (1987) Down-regulation of protein kinase C is due to an increased rate of degradation. Biochem. J. 244, 775–779.

Von Hoff, D. D., Forseth, B., and Warfel, L. E. (1985) se of a Radiometric System to Screen for Antineoplastic Agents: Correlation with a Human Tumor Cloning System. Cancer Res. 45, 4032–4036.

I claim:

1. A test cell in which there is present in the cell a target protein, wherein the presence of the target protein induces a change in a selected phenotypic characteristic of the test cell, other than the level of the target protein in the test cell, per se, wherein the change in the selected phenotypic characteristic is comparatively greater in the test cell than in a control cell, and wherein the change in the selected phenotypic characteristic has been found to be responsive to treatment with a test substance that is capable of inhibiting or activating a biochemical function of the target protein, by a method comprising the steps of:

a) providing a test cell in which a target protein is present at a higher level relative to a control cell which produces the protein at a lower level or essentially does not produce the protein;

b) treating the test cell of step (a) containing the target protein of step (a) with a test substance; and

c) examining the treated test cell of step (b) to determine whether an selected phenotypic characteristic that is responsive to inhibition or activation of the target protein of step (a) has been altered by treatment with the test substance of step (b).

* * * * *

(2)

No. 04-695

Supreme Court, U.S.
FILED
DEC 17 2004
OFFICE OF THE CLERK

IN THE

Supreme Court of the United States

HOUSEY PHARMACEUTICALS, INC.,
Petitioner,

v.

ASTRAZENECA UK LTD., *et al.*,
Respondents.

ON PETITION FOR A WRIT OF CERTIORARI TO THE UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

BRIEF FOR RESPONDENTS IN OPPOSITION

ROBERT BAECHTOLD
FITZPATRICK CELLA HARPER & SCINTO
30 Rockefeller Plaza
New York, NY 10112-3801
(212) 218-2100
Counsel for Bristol-Myers Squibb Company

SETH P. WAXMAN
Counsel of Record
WILMER CUTLER PICKERING HALE AND DORR LLP
2445 M Street, N.W.
Washington, DC 20037
(202) 663-6000
Counsel for Wyeth and Wyeth Pharmaceuticals, Inc.

HERBERT F. SCHWARTZ
FISH & NEAVE LLP
1251 Avenue of the Americas
New York, NY 10020
(212) 596-5000
Counsel for AstraZeneca UK Ltd., AstraZeneca LP, AstraZeneca Pharmaceuticals LP and Aventis Pharmaceuticals, Inc.

JOHN F. LYNCH
HOWREY SIMON ARNOLD & WHITE, LLP
750 Bering Drive
Houston, TX 77057-2198
(713) 787-1400
Counsel for Merck & Co., Inc.

QUESTION PRESENTED

Whether the court of appeals properly agreed with the district court's construction of a disputed claim term common to petitioner's patents, and accordingly affirmed the district court's judgment that the patents are invalid and not infringed.

CORPORATE DISCLOSURE STATEMENT

The following are parent companies of, or publicly held companies that own 10% or more of the stock of, respondents AstraZeneca UK Ltd., AstraZeneca LP, and AstraZeneca Pharmaceuticals LP: AstraZeneca PLC, AstraZeneca Treasury Ltd., and AstraZeneca AB.

The following are parent companies of, or publicly held companies that own 10% or more of the stock of, respondent Aventis Pharmaceuticals, Inc.: Sanofi-Aventis, Aventis SA, Aventis Inc., Aventis Holdings Inc., Aventis Beteiligungsverwaltung GmbH, Aventis Pharma SA, Aventis Investissement, and Aventis Pharma Holdings GmbH.

Bristol-Myers Squibb Company has no parent company, and no publicly held company holds 10% or more of its stock.

Merck & Co., Inc., has no parent company, and no publicly held company holds 10% or more of its stock.

Wyeth Pharmaceuticals, Inc., is a wholly-owned subsidiary of Wyeth. Wyeth has no parent company, and no publicly held company holds 10% or more of its stock.

TABLE OF CONTENTS

	Page
QUESTION PRESENTED	i
CORPORATE DISCLOSURE STATEMENT	ii
TABLE OF AUTHORITIES	iv
OPINIONS BELOW	1
STATEMENT	2
ARGUMENT	5
CONCLUSION	7

TABLE OF AUTHORITIES

CASES

Page(s)

Bayer AG v. *Housey Pharmaceuticals, Inc.*, 340 F.3d 1367 (Fed. Cir. 2003) 2

Bayer AG v. *Housey Pharmaceuticals, Inc.*, 169 F. Supp. 2d 328 (D. Del. 2001), *aff'd*, 340 F.3d 1367 (Fed. Cir. 2003) 2

Bayer AG v. *Housey Pharmaceuticals, Inc.*, No. Civ-01-148-SLR, 2003 WL 22953817 (D. Del. Dec. 4, 2003), *appeal pending*, No. 04-1194 (Fed. Cir.) (argued Nov. 3, 2004) 1, 2, 3, 6

Bayer AG v. *Housey Pharmaceuticals, Inc.*, No. Civ.-01-148-SLR, 2004 U.S. Dist. LEXIS 9633 (D. Del. May 4, 2004), *appeal pending*, No. 04-1403 (Fed. Cir.) (argument scheduled for Jan. 13, 2005) 1, 3, 6

Dayco Products Inc. v. *Total Containment, Inc.*, 329 F.3d 1358 (2003) 6

Phillips v. *AWH Corp.*, 376 F.3d 1382 (Fed. Cir. 2004) 5

Precision Instrument Manufacturing Co. v. *Automotive Maintenance Machinery Co.*, 324 U.S. 806 (1945) 6

STATUTES AND RULES

35 U.S.C. § 271(g) 2

35 U.S.C. § 285 3

S. Ct. Rule 12.6 3

IN THE

Supreme Court of the United States

No. 04-695

HOUSEY PHARMACEUTICALS, INC.,

Petitioner,

v.

ASTRAZENECA UK LTD., *et al.*,

Respondents.

ON PETITION FOR A WRIT OF CERTIORARI TO THE UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

BRIEF FOR RESPONDENTS IN OPPOSITION

OPINIONS BELOW

The opinion of the court of appeals (Pet. App. 1a-22a) is reported at 366 F.3d 1348. The order of the district court (Pet. App. 23a) entering a stipulated judgment holding petitioner's patents invalid and not infringed on the basis of claim constructions adopted after a joint hearing in this case and *Bayer AG* v. *Housey Pharmaceuticals, Inc.*, Civ. No. 01-148-SLR (D. Del.), and the claim construction order formally entered in *Bayer* (Pet. App. 30a-32a), are not reported. The district court's later opinions in *Bayer* holding the same patents unenforceable because of Dr. Housey's inequitable conduct during their prosecution, and awarding Bayer $1.8 million in attorneys' fees, are not officially reported, but they are available at 2003 WL 22953187 and 2004 U.S. Dist. LEXIS 9633, respectively.

STATEMENT

This is one of a number of cases involving four patents obtained by Gerard Housey and assigned to petitioner Housey Pharmaceuticals, Inc. The patents purport to cover methods for pharmaceutical research. The litigation has not gone well for petitioner.

1. In the first of two proceedings relevant here, Bayer AG and its U.S. affiliate Bayer Corporation sued petitioner for a judgment declaring the Housey patents invalid, unenforceable and not infringed. *See Bayer AG* v. *Housey Pharms., Inc.*, 169 F. Supp. 2d 328, 328 (D. Del. 2001). The district court rejected one of petitioner's theories of infringement (based on 35 U.S.C. § 271(g)) as a matter of law. *Id.* at 330-331. The court later entered final judgment on that issue under Fed. R. Civ. P. 54(b), and the Federal Circuit affirmed. *Bayer AG* v. *Housey Pharms., Inc.*, 340 F.3d 1367, 1370, 1378 (Fed. Cir. 2003).

While that infringement decision was on appeal, the district court consolidated *Bayer* with the present case for purposes of construing disputed claim language in the patents. *See* Pet. App. 2a n.1. Its construction led, as petitioner stipulated, to the conclusion that the patents are invalid and not infringed. *Id.* at 30a-32a (claim-construction order), 24a-29a (stipulation).

The court later conducted a four-day trial in *Bayer* to decide whether Dr. Housey had engaged in "inequitable conduct" while prosecuting his patents before the Patent and Trademark Office (PTO). The court concluded that Housey's conduct showed "a pattern of behavior inconsistent with [his] duty of candor" to the PTO; that his testimony before the court was not credible; and that Bayer had proven by clear and convincing evidence that Housey never performed experiments that he described in his patent applications and relied on in overcoming an initial rejection by the patent examiner. *Bayer AG* v. *Housey Pharms., Inc.*, No. Civ-01-148-SLR, 2003 WL 22953817, ¶¶ 109, 115-116 (D. Del. Dec. 4, 2003) (*Bayer III*), *appeal pending*, No. 04-1194 (Fed. Cir.) (argued Nov. 3, 2004). Because "[i]ntentionally misrep-

resenting key experiments to the PTO is conduct which cannot be explained, defended nor excused," the court held that, under established law, Housey's inequitable conduct during the prosecution rendered all four of his patents unenforceable. *Id.* ¶ 117. In a separate order, the court held that Bayer's case was an "exceptional" one within the meaning of 35 U.S.C. § 285, and exercised its discretion to order petitioner to pay Bayer $1.8 million in attorneys' fees. *Bayer AG* v. *Housey Pharms., Inc.*, No. 01-148-SLR, 2004 U.S. Dist. LEXIS 9633 (D. Del. May 4, 2004) (*Bayer IV*), *appeal pending*, No. 04-1403 (Fed. Cir.) (argument scheduled for Jan. 13, 2005).

2. In the present proceeding, petitioner sued respondents and others in the same district court in which *Bayer* was pending, alleging that respondents were infringing the same four patents. Pet. App. 2a.[1] As noted above, the court consolidated the two cases solely for purposes of construing the patent claims. *See id.* at 2a n.1. Based on its construction of the claim term "inhibitor or activator of a protein," and petitioner's stipulation about the effect of that construction, the court held the patents invalid and not infringed. *Id.* at 2a, 23a.[2]

---

[1] The petition names Roche Holdings, Inc., Hoffman-La Roche Inc., Roche Laboratories, Inc., and Syntex (U.S.A.) Inc. as respondents. Those entities settled with petitioner while this case was pending in the Federal Circuit, and stipulated to dismissal of the appeal (and a cross-appeal) as to them. The court of appeals denied the motion on the ground that there was no separately-docketed proceeding that could be dismissed, but it revised the official caption of the case "to reflect that Roche will not be participating in this appeal." C.A. Order of Mar. 5, 2003, at 2 (Fed. Cir. No. 03-1193); *see* Pet. App. 1a (listing Roche entities as "defendants" but not as "appellees" or "cross-appellants"). The Roche entities accordingly ceased to be parties in the court below, are not proper respondents in this Court, and in any event would appear to "have no interest in the outcome of the petition." S. Ct. Rule 12.6.

[2] Based on its construction of the term "phenotypic characteristic," and petitioner's stipulation about the effect of that construction, the court also held the patents not infringed by the AstraZeneca, Merck, and Bristol-Myers respondents. *See* Pet. App. 25a-26a. The Federal Circuit did

The Federal Circuit affirmed. Pet. App. 1a-22a. In particular, the court rejected petitioner's argument that the term "inhibitor or activator of a protein" (*id.* at 9a), read in the context of the other claim language, the patent specification, and the prosecution history, should be limited to substances that inhibit or activate a protein's biological activity in a particular way—*i.e.*, by binding directly to it. *Id.* at 8a-13a. Among other points, the court noted that "the word 'bind' or 'binding' appears nowhere in the '281 patent in conjunction with" the disputed claim term. *Id.* at 9a. Similarly, it rejected petitioner's reliance on "the recurring presence of the terms 'specific' and 'specifically' in the specification and the prosecution history," both because they gave rise to no strong inference of a direct-binding limitation, and because of "the absence of the word 'specific' in the claim language [itself] (and [the court's] repeated warnings not to import limitations from the specification that are not present in the claims[.]" *Id.* at 11a-12a.

Judge Newman dissented, generally arguing that the majority should have placed less weight on the plain meaning of terms used in the patent claims. Pet. App. 15a-19a. Focusing on the word "specificity" (although that word does not appear in the claims), Judge Newman considered the patents ambiguous on their face, and the technical evidence conflicting. *Id.* at 19a-22a. She would have remanded the matter to the district court for further claim-construction proceedings. *Id.* at 22a.

The full court denied petitioner's request for rehearing *en banc*, without recorded dissent. Pet. App. 33a-34a.

---

not find it necessary to address the construction of "phenotypic characteristic."

ARGUMENT

Petitioner devotes much of its submission to rearguing the merits of its proposed claim construction, which has been rejected by both the district court and the Federal Circuit. *See* Pet. 1-12, 22-30.[3] Ultimately, however, petitioner does not ask this Court to review that patent-specific question. Instead, it argues (Pet. 14) that "[c]onflicts among Federal Circuit decisions establish the need for clarification of the proper approach to construing patent claims."

As petitioner notes (Pet. 15-17), however, the Federal Circuit itself recently granted *en banc* review in a different case in order to address that very question. *See Phillips* v. *AWH Corp.*, 376 F.3d 1382 (Fed. Cir. 2004) (*per curiam*) (*en banc*). Once the Federal Circuit has decided *Phillips*—with the benefit of "the roughly three dozen amicus briefs, including one by the United States, that have been filed to address these fundamental questions" (Pet. 17)—this Court will have ample opportunity to consider whether it should review, either in *Phillips* or in a later case, whatever approach the Federal Circuit adopts. In the meantime, there is no reason for the Court to delay resolution of the present case.[4]

---

[3] For present purposes we see no need to respond to petitioner's arguments on the merits. We note, however, that while patent holders typically seek expansive readings of their claims, here petitioner seeks to distinguish prior art and avoid invalidity by importing into the claims an unstated limitation requiring that the "inhibitor or activator of a protein" identified by the claimed methods must act by binding directly to the protein. Yet, as the court of appeals pointed out, "[t]he word 'bind' or 'binding' appears nowhere in the [representative] patent in conjunction with an 'inhibitor or activator of a protein.'" Pet. App. 9a; *see also id.* at 12a ("specific" does not appear in claim language). Petitioner characterizes the construction adopted by both courts below as "bizarre[]" and senseless. Pet. 13. Yet even Judge Newman, although critical of the majority's approach, did not accept *petitioner's* claim construction. *See* Pet. App. 19a-22a. She concluded only that the claims were ambiguous, and would have "remand[ed] for resolution of conflicting scientific evidence." *Id.* at 22a.

[4] The Federal Circuit granted *en banc* review in *Phillips* on July 21, 2004. 376 F.3d at 1382. Petitioner's request for rehearing *en banc* in this case was filed on May 21, 2004, and denied on August 25, 2004. *See* Pet.

There is, moreover, a further consideration, not noted in the petition. As explained above (at 2-3), in the parallel *Bayer* proceeding, the same district court that decided this case also found, after a four-day trial and applying the clear-and-convincing-evidence standard, that in obtaining the patents at issue here Dr. Housey made "material misrepresentations [to the PTO] regarding experiments he claimed to have conducted," "knowingly withheld prior art," and "engaged in a pattern of concealment inconsistent with the duty of candor" on the part of patent applicants. *Bayer IV*, 2004 U.S. Dist. LEXIS 9633, at *2-3 (attorneys' fees decision, describing earlier findings); *see Bayer III*, 2003 WL 22953187, ¶¶ 94-117. Based on those findings, the court applied the inequitable conduct doctrine and held all four patents unenforceable. *Bayer III*, 2003 WL 22953187, ¶ 117; *see, e.g.*, *Precision Instrument Mfg. Co.* v. *Automotive Maint. Mach. Co.*, 324 U.S. 806, 814-820 (1945); *Dayco Prods., Inc.* v. *Total Containment, Inc.*, 329 F.3d 1358, 1362-1364 & n.3 (Fed. Cir. 2003) (discussing doctrine).

The inequitable conduct decision in *Bayer III* is currently on appeal. *Bayer AG* v. *Housey Pharms., Inc.*, No. 04-1194 (Fed. Cir.) (argued Nov. 3, 2004).[5] Unless the Federal Circuit reverses the district court's holding that the patents are unenforceable, any dispute about the proper construction of the patents' claims is moot. Accordingly, this case would not be an appropriate vehicle for review of general claim-construction principles by this Court, even if

---

App. 34a; C.A. Docket (No. 03-1193). The court of appeals accordingly had ample opportunity to evaluate this case as a vehicle for addressing general claim-construction issues, and chose instead to grant review in *Phillips*. Moreover, the Federal Circuit evidently saw no reason to hold this case pending its decision in *Phillips*. *Cf. Novartis Pharms. Corp.* v. *Eon Labs Mfg., Inc.*, No. 03-1211 (Fed. Cir.) (petition for rehearing *en banc* filed Apr. 16, 2004, and still pending); *Nystrom* v. *Trex Co., Inc.*, No. 03-1092 (Fed. Cir.) (petition for rehearing *en banc* filed July 26, 2004, and still pending). There is no reason for this Court to take a different approach.

[5] Petitioner's counsel of record in this Court also presented the oral argument for petitioner in the *Bayer III* appeal.

those issues were otherwise ripe for such review. In any event, the inequitable conduct proceedings make clear that this case provides no occasion for the Court to depart from its usual practices by, as petitioner suggests (Pet. 29-30), holding the present petition pending further developments in a different case in the court of appeals.

CONCLUSION

The petition for a writ of certiorari should be denied.

Respectfully submitted,

ROBERT BAECHTOLD
FITZPATRICK CELLA HARPER & SCINTO
30 Rockefeller Plaza
New York, NY 10112-3801
(212) 218-2100
Counsel for Bristol-Myers Squibb Company

SETH P. WAXMAN
Counsel of Record
WILMER CUTLER PICKERING HALE AND DORR LLP
2445 M Street, N.W.
Washington, DC 20037
(202) 663-6000
Counsel for Wyeth and Wyeth Pharmaceuticals, Inc.

HERBERT F. SCHWARTZ
FISH & NEAVE LLP
1251 Avenue of the Americas
New York, NY 10020
(212) 596-5000
Counsel for AstraZeneca UK Ltd., AstraZeneca LP, AstraZeneca Pharmaceuticals LP and Aventis Pharmaceuticals, Inc.

JOHN F. LYNCH
HOWREY SIMON ARNOLD & WHITE, LLP
750 Bering Drive
Houston, TX 77057-2198
(713) 787-1400
Counsel for Merck & Co., Inc.

DECEMBER 2004

(3)

No. 04-695

IN THE

Supreme Court of the United States

HOUSEY PHARMACEUTICALS, INC.,
Petitioner,

v.

MERCK & CO., INC., ASTRAZENECA UK LTD., ASTRAZENECA LP, ASTRAZENECA PHARMACEUTICALS LP, AVENTIS PHARMACEUTICALS, INC., BRISTOL-MYERS SQUIBB COMPANY, ROCHE HOLDINGS, INC., HOFFMANN-LA ROCHE INC., ROCHE LABORATORIES, INC., SYNTEX (U.S.A.) INC., WYETH, and WYETH PHARMACEUTICALS INC.,
Respondents.

On Petition for a Writ of Certiorari to the United States Court of Appeals for the Federal Circuit

---

REPLY BRIEF FOR PETITIONER

R. TERRANCE RADER
JAMES F. KAMP
RADER, FISHMAN & GRAUER, PLLC
39533 Woodward Ave.
Suite 140
Bloomfield Hills, MI 48304
(248) 594-0600

RICHARD G. TARANTO
(Counsel of Record)
FARR & TARANTO
1220 19th Street, NW
Washington, DC 20036
(202) 775-0184

WILSON-EPES PRINTING CO., INC. – (202) 789-0096 – WASHINGTON, D. C. 20001

TABLE OF CONTENTS

	Page
TABLE OF AUTHORITIES	ii
ARGUMENT	2
CONCLUSION	10

TABLE OF AUTHORITIES

CASES	Page
Akron Polymer Container Corp. v. Exxel Container, Inc., 148 F.3d 1380 (Fed. Cir. 1998)	9
Allied Colloids Inc. v. American Cyanamid Co., 64 F.3d 1570 (Fed. Cir. 1995)	9
Bayer AG v. Housey Pharms., Inc., 2003 WL 22953817, (D. Del.), *appeal argued Nov. 3, 2004* (Fed. Cir. No. 04-1194)	7
CFMT, Inc. v. Yieldup Int'l Corp., 349 F.3d 1333 (Fed. Cir. 2003)	9
Dayco Prods., Inc. v. Total Containment, Inc., 329 F.3d 1358 (Fed. Cir. 2003)	9
Florida State Bd. Of Educ. v. American Bioscience, Inc., 333 F.3d 1330 (Fed. Cir. 2003)	9
Gambro Lundia AB v. Baxter Healthcare Corp., 110 F.3d 1573 (Fed. Cir. 1997)	9
Halliburton Co. v. Schlumberger Technology Corp., 925 F.2d 1435 (Fed. Cir. 1991)	9
Hoffmann-La Roche, Inc. v. Promega Corp., 323 F.3d 1354 (Fed. Cir. 2003)	9
Juicy Whip, Inc. v. Orange Bang, Inc., 292 F.3d 728 (Fed. Cir.), *cert. denied*, 537 U.S. 1019 (2002)	9
Kingsdown Med. Consultants, Ltd. v. Hollister, Inc., 863 F.2d 867 (Fed. Cir. 1988)	9
Markman v. Westview Instruments, Inc., 517 U.S. 370 (1996)	3
Molins PLC v. Textron, Inc., 48 F.3d 1172 (Fed. Cir. 1995)	9
National R.R. Passenger Corp. v. Pennsylvania P.U.C., 288 F.3d 519 (3d Cir. 2002), *cert. denied*, 538 U.S. 1057 (2003)	10
Phillips v. AWH Corp., 376 F.3d 1382 (Fed. Cir. 2004)	2, 3, 5, 6

TABLE OF AUTHORITIES

	Page
United States v. Adams, 383 U.S. 39 (1966)	2
United States v. Munsingwear, 340 U.S. 36 (1950)	8
Restatement Second of Judgments § 28(2).....	10

IN THE

Supreme Court of the United States

No. 04-695

HOUSEY PHARMACEUTICALS, INC.,
Petitioner,

v.

MERCK & CO., INC., ASTRAZENECA UK LTD., ASTRAZENECA LP, ASTRAZENECA PHARMACEUTICALS LP, AVENTIS PHARMACEUTICALS, INC., BRISTOL-MYERS SQUIBB COMPANY, ROCHE HOLDINGS, INC., HOFFMANN-LA ROCHE INC., ROCHE LABORATORIES, INC., SYNTEX (U.S.A.) INC., WYETH, and WYETH PHARMACEUTICALS INC.,
Respondents.

On Petition for a Writ of Certiorari to the United States Court of Appeals for the Federal Circuit

REPLY BRIEF FOR PETITIONER

Respondents' opposition leaves wholly untouched the affirmative case for certiorari: the need for clarification of claim-construction principles, the conflicts over such principles that bear directly on the ruling in this case, and the fact that the patent-destroying construction adopted in this case violates the most fundamental command of proper claim construction, *i.e.*, to adopt a sensible reading that captures the invention. Instead, respondents briefly cite two reasons for denying review. Both are meritless and embody improper

disregard of the importance of preserving patent rights—here, rights that are vitally important to a tiny company, have been licensed by a majority of the world's major research-based pharmaceutical companies, and have contributed to significant advances in recent cancer treatments. Pet. 2-3.

ARGUMENT

I. Respondents' opposition does not take issue with any of the key affirmative reasons for granting review and correcting the decision in this case—either now or after awaiting the Federal Circuit's decision in *Phillips v. AWH Corp.*, 376 F.3d 1382 (Fed. Cir. 2004) (granting *en banc* review of various claim-claim construction issues), which is scheduled for oral argument on February 8, 2005.

A. Respondents do not dispute that there is a need for clarification of fundamental claim-construction principles. The Federal Circuit's own grant of *en banc* review in *Phillips* (reflecting a host of patent-law commentaries) makes any such dispute impossible. Pet. 15-16. Nor do respondents dispute that the decision in this case directly implicates the claim-construction issues set for review in *Phillips* and doctrinal conflicts among Federal Circuit precedents, including on (1) the impermissibility of constructions that render claim language circular or meaningless surplusage, (2) the central importance to a sound claim construction of attending to patents' explanation of the limits of prior art and how the claimed invention overcomes such limits, and (3) the significance of the principle that patent-invalidating constructions be avoided if possible. Pet. 14-15. Finally, respondents nowhere disagree with the petition's showing that a proper context-dependent, substance-driven approach to claim construction—generally supported by the United States as *amicus* in *Phillips*—is found in this Court's precedents, which call for avoidance of invalidating constructions and an approach to claim construction that is centrally focused on "ascertaining the invention" (*United States v. Adams*, 383

U.S. 39, 49 (1966)) through a "necessarily sophisticated analysis of the whole document" (*Markman v. Westview Instruments, Inc.*, 517 U.S. 370, 389 (1996)). *See* Pet. 17-22. In short, the need for review, and ready availability of proper standards for re-establishing a sound approach to claim construction, stand unrebutted.

B. Respondents include all of one footnote in their brief addressing the merits of the ruling in this case. Opp. 5 n.3. That short discussion says nothing to dispute that the ruling here illustrates the very errors of claim-construction principle featured by the petition (and at issue in *Phillips*). It also says literally nothing to rebut the petition's showing that the construction adopted by the majority (1) contradicts ordinary usage and common sense and renders claim language circular and meaningless surplusage, (2) overrides the patents' explanation of the essential advance over prior art, and (3) treats the invalidity-avoidance principle as an insignificant afterthought. Pet. 22-29.

As to *the majority's adopted construction*, respondents note the petition's showing that it is senseless, then immediately change the subject to the merits of *petitioner*'s alternative construction. Opp. 5. They give no answer at all to the showing that the majority's (and district court's) construction produces a nonsensical tautology, contradicting ordinary usage by making the method of screening into a definition of what is being screened for, with perfection attributed to the screening test by *definition* (everything showing the differential effect in the assay is an "inhibitor or activator of a protein"; everything not, not). Construing the assay as definitionally perfect is outlandish in the experimental sciences, where perfection is essentially impossible—as wrong as *defining* "perpetrator" by whoever is picked out of a lineup (or "time" by the result of an occasionally faulty clock). Not surprisingly, the majority's construction contradicts the plain scientific meaning of "method of determining"—adopted by the district court and unchallenged by the Merck group of

respondents—as only indicating the searched-for result by a high probability, not perfection. Pet. 22-24. The majority's (district court's) construction is thus wholly undefended. Yet respondents do not and cannot disagree that the stipulation of invalidity and non-infringement depended entirely on *that* construction, so that error in that construction precluded the Federal Circuit's affirmance of the district court's judgment, regardless of what other construction might be considered. Pet. 8, 22.

As to petitioner's construction, respondents just baldly declare that binding to (directly interacting with) the protein molecule is somehow extraneous to the "inhibitor or activator of a protein" claim limitation. Opp. 5. That declaration wholly ignores the compelling reasons to conclude otherwise. Dictionary definitions show one standard *meaning* of "inhibiting"/"activating" an enzyme to be attaching to the molecule (to affect its action), and there is not the slightest reason for that meaning to be unavailable for proteins generally. Pet. 25. Expert testimony on the claim-construction issue, including from petitioner's adversary, confirms that understanding. Pet. 25.[1] The essence of the patents' distinction of prior-art

---

[1] Because respondents have drawn into this case the later proceedings in the *Bayer AG v. Housey Pharms., Inc.* case (Opp. 2, 6)—and Bayer was respondents' joint adversary of petitioner for claim construction—we note that petitioner's construction of "inhibitor or activator of a protein" was directly supported there by Bayer's expert Dr. Fields and Bayer's witness Dr. Hsiao. 12/09/02 Transcript at 822 (Dr. Hsiao: "Q: Okay. Now, did you understand Dr. Housey's patent to be directed to determining whether substances would bind to specific protein? A: Yes."); 12/03/02 Transcript at 144-45 (Dr. Fields) ("Q: Did you understand when you read Dr. Housey's patents in connection with your preparation work for your opinions here Dr. Housey was claiming an ability to identify inhibitors or activators that would directly interact with a specific protein? A: That's my understanding."); *id.* at 61-62, 148-49 (Dr. Fields). Dr. Fields noted "the central ten[et] of pharmacology, which is that for a substance to act on a particular protein or molecule, it needs to first bind to that molecule." *Id.* at 55.

screening methods, moreover, is precisely that they cannot and do not, whereas the newly claimed method can and does, distinguish those substances that alter cellular characteristics by directly interacting with the protein molecule from substances that alter cellular characteristics "'by other mechanisms,'" through action "'upon other cell metabolites.'" Pet. 26. Indeed, Dr. Housey explained to the PTO that "'target protein specificity . . . is the *essential point of the present invention.*'" Pet. 29 (emphasis added). Respondents have no answer for any of these compelling reasons that, not only is the construction adopted by the majority below incorrect, but petitioner's construction soundly captures the claimed invention and is accordingly correct.

II. Respondents make only two arguments against review. Both lack merit.

A. Respondents' first brief ground for opposing certiorari is that the *en banc* Federal Circuit in *Phillips* is currently considering how to resolve the conflicts of claim-construction approach and "there is no reason for the Court to delay resolution of the present case." Opp. 5. But the conclusion does not follow from the premise. Indeed, respondents' submission turns the relevance of *Phillips* on its head and ignores the most elementary demand of fairness.

A denial of certiorari here would be a final judgment of invalidity of Housey's patents and their non-infringement by respondents. Yet respondents nowhere dispute that a different outcome in this case could easily be required by the *Phillips* court's resolution of the wide range of claim-construction issues it is considering; indeed, they recognize that *Phillips* is considering the "very question" presented here. Opp. 5. If *Phillips* were already decided, an opinion that affected the proper analysis of this case—as respondents accept is entirely possible—would require equal treatment of this case, whether through a grant of review here, through a GVR in light of *Phillips*, or through a decision to hold the

petition while this Court reviews *Phillips* itself and then act in accordance with the result of that review. The accident of timing, when a decision in *Phillips* is known to a certainty to be forthcoming, should not produce a different result, terminating petitioner's patent rights when a short delay could preserve those rights.

There are only two possibilities for the effect of *Phillips*. *See* Pet. 29-30. If *Phillips* does not articulate claim-construction principles that require a reconsideration of the ruling in the present case, then review in this case is warranted because the fundamental defects in claim-construction approach on which the ruling here is based will have gone uncorrected. If *Phillips* does articulate claim-construction principles that require reconsideration of the ruling here, then those principles should be applied to this case. This Court should reject respondents' casual dismissal of the interest in correct patent interpretation and fair treatment on which petitioner's patent rights depend.

Petitioner is hardly a mammoth pharmaceutical company with vast numbers of patents, any small number of which might be unimportant. The patents at issue are petitioner's principal assets. Cavalier treatment of the interest in giving proper scope to such patents is inconsistent with patent law's commitment to protecting small commercial entities, not just large commercial ventures.

B. Respondents' second ground for opposing review is entirely extraneous to this petition. Respondents point to the district court's holding of unenforceability in a different case, which is currently on appeal. Opp. 6, citing *Bayer AG v. Housey Pharms., Inc.*, No. 04-1194 (Fed. Cir.) (argued Nov. 3, 2004). Respondents assert that unless the Federal Circuit reverses the district court ruling in *Bayer*, "any dispute about the proper construction of the patents' claims is moot." Opp. 6. This argument is so obviously incorrect that its only residual function in the opposition is one the Court should ignore:

the atmospheric one of tarring petitioner and Dr. Housey, who can hardly explain in this reply brief why the charge of inequitable conduct is so grossly wrong.[2]

---

[2] What the district court found to constitute inequitable conduct was the alleged making up of an experiment reported in the patents (Table 3), *Bayer AG v. Housey Pharms., Inc.*, 2003 WL 22953817, ¶¶ 115-117 (D. Del.), *appeal argued Nov. 3, 2004* (Fed. Cir. No. 04-1194). *Contra* Opp. 5. The court centrally relied on its negative view of three other matters, not as inequitable conduct itself, but as reasons not to believe Dr. Housey about his performance of the experiment. Bayer so recognized at oral argument in the Federal Circuit: "secrecy, failing to submit prior art, failing to credit colleagues, those were used as a basis for a credibility determination, not to find inequitable conduct. [The district judge] was very explicit to indicate that she was not finding inequitable conduct on the basis of those points. She simply said these points influence my view of Dr. Housey's credibility . . . ." Housey's appeal in the *Bayer* case directly challenges every aspect of the inequitable-conduct ruling.

Some of the district court's errors are as follows: *First*, the court said there was no documentary corroboration of the experiment, although there plainly was (a date-stamped report of the experimental results). *Id.* ¶ 116. *Second*, the court made clearly erroneous findings about what equipment was available in the laboratory (*id.* ¶¶ 56, 57)—findings urged by Bayer to the district court but in fact lacking any evidentiary support and conclusively disproved by the testimony of Bayer's own witnesses and contemporaneous laboratory notebooks of Bayer witnesses. *Third*, the court carefully listed three reasons for not crediting Dr. Housey's testimony about his experiment, but all three were legally and/or factually incorrect. ¶ 115. **(a)** The court said that Dr. Housey failed to disclose two articles to the PTO, when the articles are disclosed on the face of the patent applications (¶¶ 77, 84) and when, in any event, the court expressly found Dr. Housey did not believe the articles to be material, the essential precondition to disclosure (¶¶ 73, 81). **(b)** The court noted an unidentified failure to acknowledge the contributions of colleagues reflected in a *Cell* journal paper, despite the fact that the paper was submitted to the PTO and on its face discloses the colleagues' contributions (¶¶ 34, 46) and when the only relevant *patent-law* issue with colleagues' contributions was possible co-inventorship, yet the putative co-inventors *conceded* that they were not co-inventors of the invention as Dr. Housey genuinely understood it (¶¶ 85, 86). **(c)** Most importantly (because the court said this was the backdrop for its other assessments, ¶ 107), the court

This case is not moot. Respondents' naked assertion of present mootness not only is unaccompanied by any citation of precedent. It is indisputably wrong. The parties to this case have a present, live stake in the outcome of the claim-construction ruling, with its invalidity and non-infringement consequence, at least as long as the inequitable-conduct issue remains in pending litigation, as respondents recognize it is. That simple fact fully answers respondents' mootness contention. Indeed, if the case were moot now, the required result would be a vacatur of the decisions below with instructions to dismiss (subject of course to reinstatement if the inequitable-conduct ruling in *Bayer* were set aside). *United States v. Munsingwear*, 340 U.S. 36 (1950). Respondents do not suggest such a disposition.

Not only is the case obviously not moot, but there are several necessary (if not sufficient) contingencies that would have to occur (over an extended period) before the claim-construction issue presented for review could become moot. *First*, the Federal Circuit would have to affirm the district court's ruling of unenforceability for inequitable conduct in

---

fundamentally misunderstood patent law in *condemning* Dr. Housey's efforts to keep his patent-specific work secret from others in the university laboratory, when those efforts undisputedly were undertaken on advice of counsel and (as Bayer did not dispute on appeal) such pre-application secrecy is positively demanded by patent law in order to *preserve* the right to priority both abroad and in the United States (*e.g.*, 35 U.S.C. § 102 (public use or knowledge defeats novelty)). *Finally*, based upon its three legally and/or factually incorrect reasons for not crediting Dr. Housey's testimony about his experiment (which included no inconsistencies or evasiveness in the testimony), the court found that it had no alternative but to conclude that Bayer had proved by clear and convincing evidence that the Table 3 experiment was not performed, even though Bayer, with the burden of proof, did not undertake the essential step of proving the numbers *inaccurate* by attempting the very simple task of replicating the experiment. (As noted below, *see* note 4, *infra*, petitioner, after the shock of the district court's adverse finding, quickly undertook a replication and confirmed the *accuracy* of the Table 3 numbers.)

the *Bayer* case, and that ruling would have to become finally unreviewable (after any rehearing and certiorari proceedings). Petitioner has presented strong arguments in the *Bayer* appeal for setting aside the district court's inequitable-conduct ruling as infected by legal error and clearly erroneous. *See* note 2, *supra*. Respondents are aware, if only from their own citation of *Dayco Prods., Inc. v. Total Containment, Inc.*, 329 F.3d 1358 (Fed. Cir. 2003) (cited Opp. 6), that the Federal Circuit carefully examines and frequently reverses findings of inequitable conduct.[3] Accused infringers so commonly make such charges in order to "deprive patentees of their earned property rights and impugn fellow professionals" that they "have been called a 'plague' on the patent system." *Molins*, 48 F.3d at 1182.

Second, even if the inequitable-conduct ruling in the *Bayer* case were ultimately to survive, that would not settle the matter for the respondents in the present case. These respondents have no inequitable-conduct ruling in their favor: they dropped their inequitable-conduct defenses, without prejudice, to secure the final judgment of invalidity and non-infringement. Pet. App. 25a-26a. Accordingly, they would have to return to the district court in this case—should the claim-construction ruling be set aside, which automatically

---

[3] In addition to *Dayco*, cases involving complete or partial reversals of inequitable conduct determinations include *CFMT, Inc. v. Yieldup Int'l Corp.*, 349 F.3d 1333 (Fed. Cir. 2003); *Florida State Bd. Of Educ. v. American Bioscience, Inc.*, 333 F.3d 1330 (Fed. Cir. 2003); *Hoffmann-La Roche, Inc. v. Promega Corp.*, 323 F.3d 1354 (Fed. Cir. 2003); *Juicy Whip, Inc. v. Orange Bang, Inc.*, 292 F.3d 728 (Fed. Cir.), *cert. denied*, 537 U.S. 1019 (2002); *Akron Polymer Container Corp. v. Exxel Container, Inc.*, 148 F.3d 1380 (Fed. Cir. 1998); *Gambro Lundia AB v. Baxter Healthcare Corp.*, 110 F.3d 1573 (Fed. Cir. 1997); *Allied Colloids Inc. v. American Cyanamid Co.*, 64 F.3d 1570 (Fed. Cir. 1995); *Molins PLC v. Textron, Inc.*, 48 F.3d 1172 (Fed. Cir. 1995); *Halliburton Co. v. Schlumberger Technology Corp.*, 925 F.2d 1435 (Fed. Cir. 1991); *Kingsdown Med. Consultants, Ltd. v. Hollister, Inc.*, 863 F.2d 867 (Fed. Cir. 1988).

would dissolve the stipulation of invalidity and non-infringement—and then re-raise their inequitable-conduct defense. They have no such claim in this case now, and so an inequitable-conduct ground *could* not moot the present issue until *after* review of the claim-construction ruling presented here.[4] In short, there plainly is no mootness today, and any possible mootness would occur well down the road, and only after review of the claim-construction ruling challenged in the present petition.

CONCLUSION

The petition for a writ of certiorari should be granted.

Respectfully submitted,

R. TERRANCE RADER
JAMES F. KAMP
RADER, FISHMAN & GRAUER, PLLC
39533 Woodward Ave.
Suite 140
Bloomfield Hills, MI 48304
(248) 594-0600

RICHARD G. TARANTO
(Counsel of Record)
FARR & TARANTO
1220 19th Street, NW
Washington, DC 20036
(202) 775-0184

December 28, 2004

---

[4] Presumably respondents would invoke issue preclusion to gain the benefit of any final *Bayer* ruling, but whether issue preclusion would apply, a matter for initial district court decision, is hardly a foregone conclusion, if only because preclusion here would be "inequitable" (Restatement Second of Judgments § 28(2); *National R.R. Passenger Corp. v. Pennsylvania*, 288 F.3d 519, 528 (3d Cir. 2002), *cert. denied*, 538 U.S. 1057 (2003)). The most powerful evidence that Dr. Housey in fact performed the experiment at issue (*see* note 2, *supra*) is that, after the district court rendered its shocking finding of fabrication, he had an aide conduct a replication of the experiment (insofar as he had the materials) and confirmed the accuracy of the detailed numbers reported in the patents—numbers that simply *could not* have been made up (as Bayer alleged) if they could be accurately reproduced, as they now have been.